The Role of ADP in Platelet Activation and its Signalling in a Murine Model of Acute Allergic Inflammation

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The Role of ADP in Platelet Activation and its Signalling in a Murine Model of Acute Allergic Inflammation

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Abstract

Background: Clinical studies have demonstrated platelet activation in inflammatory disorders including asthma and allergic rhinitis. This is distinct from platelet aggregation involved in the maintenance of haemostasis. Whilst signalling involved in platelet activation in haemostasis is well known, signalling pathways in activation responding to inflammatory stimuli remains unclear.

Objectives: Here I investigated whether purinergic activation of platelets in allergic inflammation is distinct from purine involvement in platelet aggregation. In the second part, stimulation of platelets with a large range of both inflammatory and haemostatic stimuli was used to investigate potential distinct differences in platelet function

Methods: Balb/c mice were sensitised to Ovalbumin (OVA) and subsequent OVA challenge. Bronchoalveolar lavage fluid was analysed for inflammatory cells and blood samples were collected and analysed for platelet activation. The role of platelet purinergic receptors and associated signalling mechanisms (RhoA) were also assessed.
Additional *in vitro* experiments were performed on isolated human platelets to investigate the impact of a range of inflammatory and haemostatic stimuli on platelet function through measurements on P-selectin expression, platelet-leukocyte conjugation, aggregation and migration.

Results: Allergen challenge induced significant increases in pulmonary leukocyte recruitment compared to sham controls ($P<0.001$).$P2Y_1$ ($P < 0.001$), but not $P2Y_{12}$ or $P2X_1$ antagonism inhibited allergen induced pulmonary leukocyte recruitment. The formation of platelet-leukocyte conjugates *in vivo* and platelet/P-selectin dependent polymorphonuclear cell migration *in vitro* was exclusively $P2Y_1$ dependent, furthermore allergen challenge induced significant increases in RhoA activity, a process which was inhibited exclusively through $P2Y_1$ receptor antagonism. Leukocyte recruitment remained significantly suppressed in thrombocytopenic mice following reinfusion of platelets treated with a $P2Y_1$ antagonist or a Rho-associated kinase inhibitor confirming a crucial role for RhoA activity downstream of platelet $P2Y_1$ receptors.
Secondly, stimulation of platelets with chemotactic stimuli such as macrophage-derived chemokine ($P < 0.01$) and stromal-cell derived factor 1α ($P < 0.001$) induced significant platelet migration without increases in P-selectin, platelet-leukocyte conjugates or aggregation. Conversely haemostatic stimuli induced increases in all measured parameters bar platelet migration

Conclusion: RhoA signalling downstream of platelet $P2Y_1$, but not $P2Y_{12}$, represents a clear dichotomy in platelet activation during allergic inflammation versus haemostasis. Furthermore, platelet activation by different stimuli (inflammatory or haemostatic) can induce differences in platelet function further emphasising a dichotomy in platelet function.

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List of Abbreviations

5-HT – 5-Hydroxy-tryptamine

AC – Adenylyl Cyclase

ACD – Acid-Citrate Dextrose

ADP – Adenosine-diphosphate

AHR – Airway Hyperresponsiveness

ALI – Acute Lung Injury

AMP – Adenosine-monophosphate

APAS – anti-platelet-anti-sera

APC – Antigen Presenting Cell

ARDS – Acute Respiratory Distress Syndrome

ASPA – Animals (Scientific Procedures) Act

ATP – Adenosine-triphosphate

BAL – Bronchoalveolar Lavage

BHR – Bronchial Hyperresponsiveness

BSA – Bovine Serum Albumin

COPD – Chronic Obstructive Pulmonary Disease

COX – Cyclooxygenase

CNS – Central Nervous System

DAG – Diacylglycerol

DMEM – Dulbecco's Modified Eagle Medium

FBS – Foetal Bovine Serum

DMSO – Dimethyl Sulfoxide

DTS – Dense Tubular System

EAR – Early Asthmatic Response

ECP – Eosinophil Cationic Protein

EDN – Eosinophil Derived Neurotoxin

EGF – Endothelial Growth Factor

EPO – Eosinophil Peroxidase

FEV_1 – Forced Expiratory Volume in One Second

FITC -Fluorescein isothiocyanate

f-MLP - Formyl-Methionyl-Leucyl-Phenylalanine

GDP – Guanine Diphosphate

GEF – Guanine Exchange Factor

GP - Glycoprotein

GPCR – G Protein Coupled Receptor

GPS – Gray Patient Syndrome

GTP – Guanine-Triphosphate

IBD – Irritable Bowel Disorderr

IFG – Insulin-like Growth Factor

ICAM - Intercellular Adhesion Molecule 1

IgE - Immunoglobulin

IL - Interlukin

IP_3 – Inositol Triphosphate

LAR – Late Asthmatic Response

LPS - Lipopolysaccharide

LT - Leukotriene

MBP – Major Basic Protein

MDC – Macrophage Derived Chemokine

MFI – Mean Fluorescence Intensity

MLCK – Myosin Light Chain Kinase

MMP – Matrix Metallaprotease

MRLC – Myosin Regulatory Light Chain

OCS – Open Canalicular System

OVA – Ovalbumin

PAF – Platelet Activating Factor

PAR – Protease Activated Receptor

PBP – Platelet Basic Protein

PBS – Phosphate Buffered Saline

PCP – Platelet Cationic Protein

PDGF – Platelet Derived Growth Factor

PE - Phycoerythrin

PEG – Polyethelene Glycol

PF-4 – Platelet Factor-4

PFA - Paraformaldehyde

PG - Prostaglandin

PI3-K – Phosphatidylionositol-3-Kinase

PIP_2 - Phosphatidylinositol 4,5-bisphosphate

PKC – Protein Kinase C

PLC – Phospholipase C

PMN - Polymorphanuclear

PPP – Platelet Poor Plasma

PRP – Platelet Rich Plasma

PSGL-1 – P-selectin glycoligand-1

RA – Rheumatoid Arthritis

RANTES – Regulated on Activation, Normal T Cell Expressed and Secreted

ROCK – Rho-associated Protein Kinase

ROS – Reactive Oxygen Species

SDF-1α – Stromal Derived Factor -1α

SEM – Standard Error of the Mean

TARC – Thymus- and activation-regulated chemokine

TGF – Transforming Growth Factor

Th2 – T Helper 2

TLR – Toll-like Receptor

TMD – Transmembrane Domain

TPH – Tryptophan Hydroxylase

TRAP – Thrombin Receptor Activating Peptide

TxA_2 – Thromboxane A_2

VCAM - Vascular cell adhesion protein

VEGF – Vascular Endothelial Growth Factor

VSM – Vascular Smooth Muscle

vWF – Von Willebrand Factor

WHO – World Health Organisation

WT – Wild-Type

B-TG – β-Thromboglobulin

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Chapter I

Introduction

1.1 Asthma and Allergic Inflammation

"Asthma is a major non-communicable disease characterised by recurrent attacks of breathlessness and wheezing, which can vary in severity and frequency from person to person. During an asthma attack, the lining of the bronchial tubes swells, causing the airways to narrow and reducing the flow of air into and out of the lungs."

The above quote is taken from the world health organisation (WHO). According to the WHO, some 235 million people currently suffer from asthma (WHO 2014). In the second half of the twentieth century worldwide prevalence of asthma has increased dramatically. However it is believed that asthma prevalence has plateaued since the late 1990s. Despite this, asthma rates in the UK remain amongst the highest across Europe, with an average of 3 deaths per day due to asthma in the UK alone (Asthma UK). Currently, the NHS spends £1.1 billion on asthma through treatment and care, whilst in 2008/2009 1.1 million working days were lost due to breathing or lung problems (Asthma UK). These figures clearly demonstrate a continued unmet need for effective long-term treatment.

Typically, asthma is characterised as a chronic disease, exacerbated by acute exposures to allergens or other irritants including cold air, exercise, particulates and chemical irritants, as well as some medications e.g. aspirin/β-blockers resulting in bronchoconstriction and subsequent inflammatory cell recruitment. Asthma is now recognised as a chronic inflammatory condition associated with changes to the architecture of the airways. The typical response to allergen provocation in asthma consists of the acute inflammatory response. This comprises of the early and late phase responses characterised by bronchospasm and contributes to the development of non-specific airway hyperresponsiveness, a state of heightened contraction of the

airway wall and increased sensitivity to spasmogens. Following the acute phase, the chronic inflammatory response is initiated which is characterised by persistent inflammatory cell recruitment and structural changes to the airway architecture (airway wall remodelling), potentially contributing to the development of airway hyperresponsivess (AHR).

The early phase response is the first stage of the allergic response following allergen provocation. Typically, this is characterised by interactions of IgE and the IgE receptor FcεRI on cells such as mucosal mast cells, dendritic cells, epithelial cells and basophils (Lambrecht and Hammad, 2010; Nakanishi, 2010; Barnes, 2011; Amin, 2012; Salazar and Ghaemmaghami, 2013), and normally occurs within the first 2 hours post provocation. This cross-linkage induces the release of pro-inflammatory mediators including histamine, leukotrienes (LTC_4), prostaglandins (PGD_2), platelet activating factor (PAF), and reactive oxygen species (ROS) (Nakanishi, 2010; Barnes, 2011; Amin, 2012) leading to bronchoconstriction, mucus secretion and vasodilation (Barnes, 2011) (**figure 1.1**).

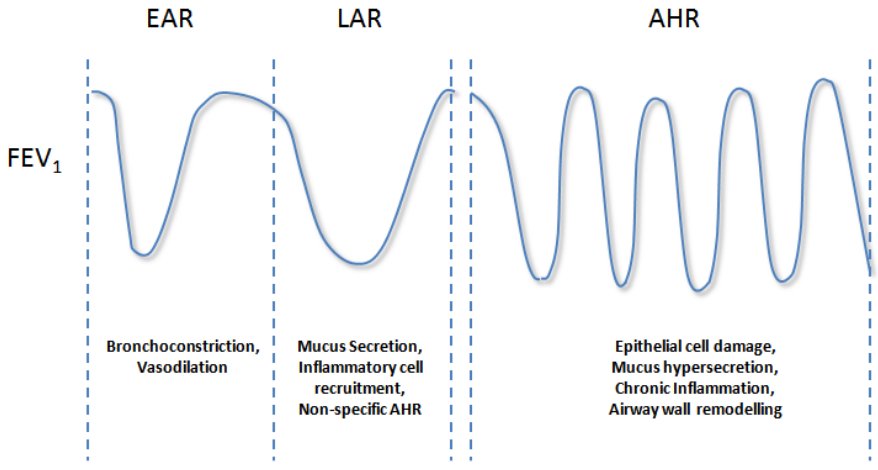


Figure 1. 1 Pathogenesis of asthma and subsequent clinical consequences

Early Asthmatic Response (EAR), Late Asthmatic Response (LAR), Airway Hyperresponsiveness (AHR), Forced Expiratory Volume (FEV_1)

6-9 hours post initial allergen challenge, the late phase of the acute response is initiated. The late phase response is typically characterised by early recruitment of inflammatory cells, in particular eosinophils to the lungs. Release of further pro-inflammatory mediators and T-cell derived cytokine occurs, including IL-4 and IL-5 (Hershey et al., 1997; Hogan et al., 1997; Moritani et al., 1998; Shi et al., 1998; Barnes, 2011). During this late phase response, inflammatory cells including eosinophils, basophils, neutrophils, macrophages and T-lymphocytes are all recruited (Wenzel et al., 1997). Once recruited, these inflammatory cells secrete further cytotxic mediators such as ROS, degradative enzymes, major basic protein (MBP), ecationic proteins, peroxidases and leukocyte derived neurotoxins. Furthermore, neutrophils, eosinophils, mast cells and basophils are all major sources of sulphidopeptide leukotrienes and PAF (Wardlaw et al., 2000), which can all contribute to further bronchospasm, vasodilation and mucus secretion. Indeed this extra bronchospasm can trigger non-specific AHR, as measured by heightened contraction of the airway wall and increased sensitivity to spasmogens (**figure 1.1**).

The chronic inflammatory response associated with asthma is characterised by the immune response with increased secretion of the Th2 cytokines (IL-4, IL-5 etc) from Th2 cells (Lambrecht and Hammad, 2010; Nakanishi, 2010; Barnes, 2011), eventually leading to alterations in tissue architecture, tissue destruction, and inappropriate repair mechanisms that might modulate lung function, leading to a state of airway hyperresponsiveness (AHR).

In patients with asthma, the $CD4^+$ subset of T cells reside within the airways where they subsequently differentiate to form T helper 2 Cells (Th2) (Ying et al., 1997). Activation of Th2 cells by APCs such as mast cells and dendritic cells triggers the release of Th2 derived chemokines such as IL-4, IL-5, IL-13, eotaxin and RANTES

(Regulated upon Activation, Normally T-cell Expressed and Secreted) (Moritani et al., 1998; Barnes, 2011; Bosnjak et al., 2011) involved in the initiation and maintenance of inflammatory cell recruitment. The importance of Th2 cells in allergic inflammation has been confirmed in murine models of allergic inflammation by the presence of severe eosinophilic airway infiltration and AHR development following adoptive transfer of TCR-transgenic Th2 cells, but not Th1 cells with subsequent challenge to the antigen Ovalbumin (OVA) (Cohn et al., 1998). Furthermore, $CD4^+$ deficient mice demonstrate impaired AHR development in models of sheep red blood cell (SRBC) or OVA induced allergic inflammation combined with a decrease in Th2 cell derived IL-4 and IL-5 secretion resulting in impaired eosinophil recruitment (Gavett et al., 1994; Garlisi et al., 1995). The importance of Th2 cell activation in eosinophil recruitment has been demonstrated in a number of studies as the Th2 derived chemokines IL-4 and IL-5 have both been implicated in pulmonary eosinophil recruitment in asthmatics (Hershey et al., 1997; Hogan et al., 1997; Shi et al., 1998). Indeed IL-4 and IL-5 deficient mice demonstrate decreased leukocyte recruitment and AHR (Coyle et al., 1995; Foster et al., 1996; Hamelmann et al., 1997; Lampinen et al., 1999). Importantly however, IL-12 has been shown to be capable of shutting down the Th2 response (Barnes, 2011). Interestingly, despite a decrease in both blood and sputum eosinophil numbers in response to allergen challenge, neither recombinant IL-12 or anti-IL-5 has shown any effect on BHR, demonstrating that the Th2 response alone is not the only process involved (Matsuse et al., 2003).

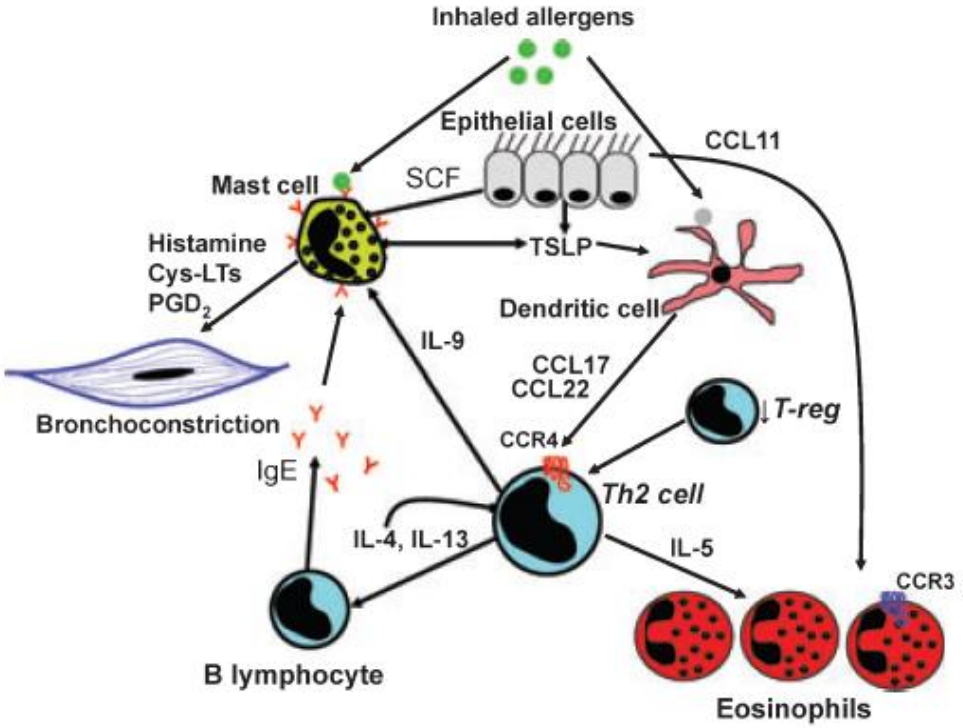


Figure 1. 2. The Immune response to allergen

During the chronic inflammatory phase, activated Th2 cells can also activate B cells inducing antibody secretion (plasma cells, a cell type late in B cell development) (Drolet et al., 2010) (**figure 1.2**). Indeed, B cells have been previously been detected in the respiratory mucosa following exposure to allergen in a murine model of allergic inflammation (Singh et al., 2008; Drolet et al., 2010) and also demonstrate an ability to behave as APCs, allowing them to drive Th2 cell responses (Lindell et al., 2008). The most widely accepted role of B cells in allergic inflammation is their ability to produce IgE (Lack et al., 1995). This process is known as immunoglobulin switching of B cells and is processed by IL-4 and IL-13 (Lack et al., 1995; Barnes, 2011). This production of IgE allows for further cross-linking of allergen with IgE on FcεRI expressing inflammatory cells inducing further release of histamine,

leukotrienes and ROS leading to bronchoconstriction and tissue damage. IgE production also demonstrates an ability to induce eosinophil recruitment as the administration of IgE specific antibodies was able to inhibit antigen induced eosinophil recruitment (Coyle et al., 1995). Importantly, deficiencies in the low affinity IgE receptor FcεRII (CD23) showed no significant difference in eosinophil recruitment when compared to wild-type mice (Riffo-Vasquez et al., 2000). Thus, it would appear that at least in the case of murine models, IgE dependent pathways co-exist with T cell dependent pathways demonstrating multiple pathways capable of inducing leukocyte recruitment and the development of AHR.

Despite the abundance of research demonstrating the efficacy of anti-IL-5, IL-5 and IL-13 therapies in murine models, these antibodies have demonstrated very poor efficacy in clinical trials with patients with asthma, with a persistent state of AHR remaining (Leckie et al., 2000). Thus, there is a continued need to research the mechanisms involved in the inflammatory response.

Current asthmatic therapies focus on combination therapies with corticosteroids e.g. fluticasone proprionate to provide inflammatory relief and long acting β_2 agonists such as salmeterol to provide bronchodilation (GINA guidelines 2013). Unfortunately, not all asthmatic patients respond to corticosteroid treatment (patients who demonstrate no objective responses/improvements to FEV_1 despite long term treatment with high doses of corticosteroids). There is clearly therefore a large unmet need for better treatments for asthmatic patients in dealing with excessive inflammation and AHR. Furthermore, current therapies are associated with unwanted side effects, depending on systematic or long term administration e.g. corticosteroids.

1.2 Platelet Development and Maturation

The platelet is a specialised component of the blood with a significant role in the cessation of bleeding following injury. The mammalian platelet is a sub cellular fragment approximately 0.5μm - 3μm in size with a resting discoid shape (Hartwig and Italiano, 2003). Platelets are produced within the cytoplasm of the megakaryocyte before being released into the circulation where they survive for 7-10 days (Hartwig and Italiano, 2003). Under normal (resting) conditions, blood platelet concentration typically ranges between 1.5 and $3x10^8$ platelets/ml (Hartwig and Italiano, 2003). Furthermore under normal haemostasis these platelets demonstrate no interaction with either the vascular endothelium or other circulating platelets.

Platelets are created through a process of maturation within the megakaryocytes, here pro-platelets are formed protuding out from the megakaryocytes. Whilst the precise mechanism remains unclear, Radley *et al* 1980 were the first to postulate the theory that long pro-platelet extensions from the megakaryocytes were intimately linked with the production and release of platelets (Radley and Scurfield, 1980). A more recent extension of the pro-platelet theory involves the production of platelets from the tips of the pro-platelets (Richardson et al., 2005). In this scenario, platelets form on the tip of the pro-platelet extrusion extending outwards from the megakaryocyte cell body. Importantly in this model the intracellular contents of the platelets must be moved from the regions of synthesis within the megakaryocytes cell body to the pro-platelets where the platelet organelles and granules can be assembled and packaged, thus completing platelet maturation and paving the way for subsequent release into the circulation (Leven et al., 1987; Tablin et al., 1990; Kosaki, 2005). It remains unclear where this process occurs as meegakaryocytes

have also been detected within the vasculature including the lung, demonstrating the potential to provide an additional organ for thrombopoesis alongside the bone marrow. Indeed intact megakaryocytes have been observed in the lungs of rabbits, rats and mice, with cytoplasmic extensions consistent with the formation of pro-platelets (Weyrich and Zimmerman, 2013), whilst platelets have been shown to be produced from circulating megakaryocytes within the pulmonary circulation (Trowbridge et al., 1982; Martin et al., 1983; Slater et al., 1985). In addition to animal models, megakaryocytes have been routinely detected in human lungs with elevated numbers observed in cases of acute lung injury (ALI), acute respiratory distress syndrome (ARDS), burns, asthma and thrombosis (Bozza et al., 2009). The potential for platelet production within the lung as a result of megakaryoycte fragmentation suggests a potential role for platelets as effector cells in a variety of pulmonary disorders (Weyrich and Zimmerman, 2013).

1.2.2. Platelet Ultrastructure

Platelets have a complicated structure which features an external and internal membrane system consisting of the surface-connected open canalicular system (OCS) (Bearer et al., 2002). The OCS acts as a reservoir of plasma membrane for platelet activation and the secretion of granular products. The spectrin membrane skeleton supports the OCS and plasma membrane through interactions with the actin cytoskeleton. The actin cytoskeleton controls the shape of the platelet, whilst closed channels of residual endoplasmic reticulum forms the dense tubular system (DTS) which is involved in calcium sequestration, and a vast array of additional different organelles are present including mitochondria, peroxisomes, lysosomes, α-granules and dense granules (Bearer et al., 2002). The α-granules and dense granules contain

a wide range of important mediators involved in regulating haemostasis, inflammation and angiogenesis listed below in table 1.1 which are crucial in platelet function following activation. (Ciferri et al., 2000; White, 2002; Italiano and Battinelli, 2009; Smyth et al., 2009)

Under normal physiological conditions, the platelet remains in its resting state indicated by it discoid shape. Upon activation the platelet undergoes a wide variety of structural and chemical changes. Following agonist induced activation, it is the ability of platelets to secrete mediators stored in their granules (table 1.1) allowing platelets to play important roles in the functional modulation of other cell types, including circulating platelets and leukocytes (Smyth et al., 2009).

α-granules	Dense granules	Lysosomal granules
Mulitmerin	*ATP* †‡	CD63 (LAMP-3) †
Adhesive glycoproteins	*ADP* †‡	Cathepsin D
Fibrinogen ‡	*Calcium* †‡	Cathepsin E
von Willebrand Factor ‡	*Serotonin* †‡	Proline Carboxypeptidase A
von Willebrand Factor propetide	*Pyrophosphate*	Proline Carboxypeptidase B
Fibronectin ‡	*GDP* ‡	β-N-acetylhexos-aminidase
Thrombospondin-1 †‡	*Magnesium*	β-D-glucuronidase
Vitronectin †‡	*Other secreted/released proteins*	β-D-galactosidase
Coagulation Factors	Protease nexin 1 ‡	α-D-mannosidase
Factor V ‡	Gas6 ‡	α-L-arabinofuranosidase
Protein S ‡	Amyloid β-protein precursor	acid phosphatase
Factor XI ‡	Tissue factor pathway inhibitor	arylsulphatase
Mitogenic Factors	Factor XIII ‡	
Platelet-derived growth factor †‡	α_2-Protease inhibitor	
Transforming growth factor-β †	Complement 1 inhibitor †‡	
Endothelial cell growth factor •	High molecular weight kininogen	
Epidermal growth factor	α_2-Macroglobin ‡	
Insulin-like growth factor-1	Vascular permeability factor	
Angiogenic factors	Interlukin-1β †	
Vascular endothelial growth factor •	Histidine-rich glycoprotein	
Platelet factor 4 (Inhibitor) • †	*Chemokines*	
Fibrinolytic inhibitors	MIP-1α (CCL3) †	
α_2-Plasmin inhibitor ‡	RANTES (CCL5) †	
Plasminogen activator inhibitor-1 †‡	MCP-3 (CCL7) †	
Albumin	Gro-α (CXCL1) †	
Immunoglobulins †	Platelet factor 4 (CXCL4) †‡	
Granule membrane-specific proteins	ENA-78 (CXCL5) †	
P-selectin (CD62P) †‡	NAP-2 (CXCL7) †‡	
GMP 33	Interlukin-8 (CXCL8) †•	
GPIb/IX/V ‡	TARC (CCL17) †	
GPIIb/IIIa ‡	CD63 (LAMP-3) †	
GPIV ‡		

† - Granular contents involved in inflammation

‡ - Granular contents involved in haemostasis

• - Granular contents involved in angiogenesis

Table 1. Platelet granular constituents (Adapted from Smyth *et al.* 2009)

1.3 Mechanisms of Platelet Activation

Platelet aggregation is an accepted function of platelets within the vasculature in response to exposed and damaged endothelium as a means to maintain hemostasis (Brass, 2010). Furthermore, inappropriate stimulation of platelet aggregation triggers thrombosis. The processes and mechanisms behind platelet activation stimulating aggregation have been extensively researched. However, more recently the role of platelets in the propagation of the inflammatory response has garnered more interest, including towards pathogen invasion, autoimmune and allergic diseases (Gresele et al., 1993; Moritani et al., 1998; Kowal et al., 2006; Pitchford et al., 2008; Boilard et al., 2010; Kraemer et al., 2010; Semple and Freedman, 2010). With regards to a range of inflammatory diseases including asthma, a key fundamental difference is the stimulation of platelet activation by inflammatory stimuli without subsequent platelet aggregation and thrombosis. Indeed, asthmatic patients actually demonstrate mild haemostatic defects (Maccia et al., 1977; Kowalska et al., 2000; Brown et al., 2013).

The mechanisms by which platelets become activated in response to different stimuli whilst sharing some similar signalling cascades and chemical events including granular secretion also demonstrate significant divergence in subsequent platelet function. For example, ADP acting via the $P2Y_1$ receptor and both Macrophage Derived Chemokine (MDC) acting via the CCR4 receptor and Stromal Derived Factor 1α (SDF-1α) via the CXCR4 receptor, signal through Gαq linked receptors with similar signalling cascades, as both trigger PLC stimulation and granular secretion. However whilst stimulation of the $P2Y_1$ receptor with ADP triggers transient platelet aggregation (Fabre et al., 1999), activation with either MDC or SDF-1α stimulates platelet chemotaxis, thus demonstrating a potential divergence in

platelet function (Kowalska et al., 2000; Kraemer et al., 2010). Any mechanism of platelet activation requires stimulation of one or more of the exposed surface receptors. The surface of platelets express a large range of receptors and signalling molecules including receptors from the G-Protein Coupled Protein family of Receptors (GPCRS) (Purine Receptors, Thromboxane Receptor, Thrombin Receptors, Prostaglandin I_2 (PGI_2) receptor and the adrenergic receptor (α_1R)), Ionotropic Receptors ($P2X_1$), the single transmembrane domanin spanning non-catalytic Toll-Like Receptors (TLR2, TLR4 and TLR9), integrins ($\alpha_{IIb}\beta_3$), Glycoprotein receptors (GPVI), Immunoglobulin receptors (i.e. FcεRI) and adhesion molecules (P-selectin, PSGL-1, CD40L, CD40 and ICAM-2) (Gear, 2001; Pitchford, 2007a; von Hundelshausen and Weber, 2007; Gachet, 2008; Amison et al., 2012). Stimulation and signalling through any one of these receptors/molecules may contribute to different products of platelet activation including granule secretion, rolling, adhesion, leukocyte interactions, leukocyte recruitment, aggregation and pathogen recognition.

One of the first measurable physiological responses following platelet stimulation is platelet shape change. Platelets change from a resting discoid shape into more rounded structures consisting of blebs and pseudopodia (Gear and Polanowska-Grabowska, 2002) . Activation to induce shape change is through stimulation of $G_{\alpha q}$ coupled receptors for example the TP, PAR1, PAR4 and $P2Y_1$ receptors (Brass, 2010). Stimulation of $G_{\alpha q}$ coupled receptors triggers activation of phospholipase C-β (PLC-β) catalysing the production of Inositol triphosphate (IP_3) and Diacyl-glycerol (DAG) from phosphatidylinositol 4,5-bisphosphate (PIP_2) (**figure 1.3**). IP_3 triggers Ca^{2+} mobilisation from intracellular stores whilst DAG triggers the activation of Protein Kinase C (PKC) which is responsible for the phosphorylation of subsequent

downstream proteins, in particular actin (Jin et al., 1998; Kunapuli, 2003; Gachet, 2006; Brass, 2010).

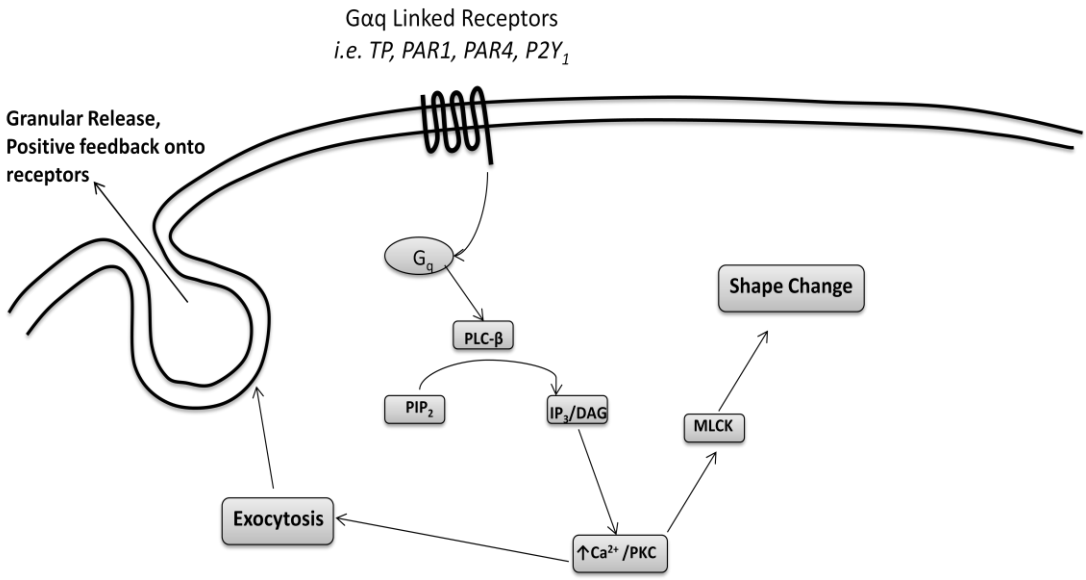


Figure 1. 3. Gq mediated induction of platelet shape change and granule secretion

Actin is a highly conserved protein of 42kDa which when polymerised forms filaments. In platelets, actin exists in 2 forms, monomeric actin (G-actin) and polymeric filamentous actin (f-actin) (Bearer et al., 2002). The process of shape change occurs over a series of steps. Under resting conditions, the discoid shape of platelets is maintained through inhibition of actin polymerisation and the sequestration of actin monomers by monomer binding proteins (thymosin β4) (Hartwig, 1992; Bearer et al., 2002), thus increasing the concentration of actin required for polymerisation. However, following stimulation the platelet contracts losing its discoid shape and becomes more spherical and slightly smaller through actin filament disassembly controlled by gelsolin in the presence of Ca^{2+} and actin

depolymerisation factors e.g. cofilin (Bearer et al., 2002). Platelet retraction is heavily dependent on interactions between Mysoin IIA and actin filaments. Platelet stimulation via either $G_{\alpha 13}$ inhibition of myosin phosphatase via RhoA activation, or via Ca^{2+} mobilisation results in increased myosin light chain kinase (MLCK) activity (Pleines et al., 2012; Aslan and McCarty, 2013). Both processes result in increased phosphorylation of myosin regulatory light chain (MRLC). MRLC phosphorylation allows the movement of myosin along actin filaments producing the contractile force involved in the rounding phase of platelet activation (Bearer et al., 2002; Li, 2002; Johnson et al., 2007). Breakdown of actin filaments presents 2 opportunities for platelet shape change. Firstly breakdown of the rigid cytoskeleton maintaining the discoid shape allows deformation of the membrane, and production of new structures within the platelet (Bearer et al., 2002). Secondly, breakdown of existing actin filaments increases the concentration of actin monomers present following their release from the ends of severed filaments (Bearer et al., 2002). These actin monomers can then bind to one of the 2 actin binding sites on the activated Arp2/3 complex (actin related protein 2/actin related protein 3 complex) (Li, 2002). The Arp2/3 complex uses its second actin binding site to bind to the sides of the actin filaments. This process allows nucleation of the actin monomers and barbed-end elongation at 70 degree angles producing the pseudopodia extending out from the platelet following the rounding phase (Mullins et al., 1998; Welch, 1998; Pantaloni, 2001; Li, 2002). As previously stated, platelet shape change is one of the first measurable physiological responses following platelet stimulation, however for continued platelet activation, release of a vast array of signalling and adhesion molecules from within the platelet granules occurs, all of which play different roles

in the mediation and regulation of the involvement of platelets in processes such as haemostasis, inflammation and angiogenesis.

In the process of granule secretion, molecules released from platelet granules, or the tissue environment provides the basis for platelet signalling. Indeed, defects in granular formation have been demonstrated in both patients and mice. Among them is Gray Patient Syndrome (GPS), a rare inherited disorder characterised by thrombocytopenia, enlarged platelets and a specific absence of α-granules (Nurden and Nurden, 2007; Blair and Flaumenhaft, 2009). Research suggests that α-granules fail to mature causing leakage of growth factors and cytokines leading to myelofibrosis as well as modification of thrombin/collagen-induced aggregation, resulting in a moderate to severe bleeding disorder (Nurden and Nurden, 2007). Other forms of α/δ-storage pool disorders (α/δ-SPD) include Hermansky-Pudlack syndrome (Cattaneo, 2002; Hurford and Sebastiano, 2008) and Chediak-Higashi syndrome (Cattaneo, 2002; Nagai et al., 2013), with both being disorders of dense granule (δ-granule) storage pool deficiency.

Out of the 3 platelet secretory granules (α, δ and lysosomes) the α-granule is the most abundant with between 50-80 granules found per platelet and a diameter of approximately 200-500nm (Cattaneo, 2002; King and Reed, 2002; Blair and Flaumenhaft, 2009). α-granules can be identified through immunofluorescent labelling of constituents such as P-selectin and vWF. As listed in table 1.1 platelet α-granules contain membrane bound proteins and proteins collected from receptor mediated endocytosis or stored following synthesis within the megakaryocyte, including the adhesion molecule P-selectin and platelet specific proteins including platelet factor 4 (PF4) and β-thromboglobulins (β-TG) (Cattaneo, 2002; Smyth et al., 2009). Whilst platelets express a substantial number of these membrane proteins

under resting conditions, platelet activation results in the release of the α-granule contents via exocytosis. This increases surface expression of the membrane proteins including integrins ($\alpha_{IIb}\beta_3$) and adhesion molecules (P-selectin), whilst also releasing substantial levels of soluble proteins such as PF-4, β-TG and growth factors (Blair and Flaumenhaft, 2009; Smyth et al., 2009) thus promoting cell-cell interactions and stimulating vascular repair.

In contrast to α granules platelets contain substantially less dense (δ) granules, with only 3-9 present in human platelets (King and Reed, 2002). The contents of dense granules function primarily for the amplification of platelet activation through secretion of additional platelet agonists such as ADP, ATP, Ca^{2+} and 5-Hydroxytryptomine (5-HT) promoting platelet aggregation. They also include a large array of chemokines capable of stimulating further platelet activation and inducing platelet and leukocyte migration including Macrophage Inflammatory Portein-1α (MIP-1α), RANTES, Thymus- and activation-regulated chemokine (TARC) (Smyth et al., 2009).

The final type of platelet secretory granule are the lysosomal (λ) granules, which have been shown to release their contents in humans post activation (Ciferri et al., 2000). Indeed lysosomal granule release requires stimulation by strong platelet agonists such as thrombin and high doses of collagen (Ciferri et al., 2000). Lysosomal granule secretion has been detected by increased concentrations of β-N-acetylhexosaminidases (HEX) (the main lysosomal glycohydrolase in blood platelets), in blood emerging from an experimental skin wound in comparison to venous blood. Importantly, levels of PF-4 and β-TG were increased demonstrating platelet activation in these regions (Ciferri et al., 2000)

Platelet granules are normally secreted in response to stimulation of platelet surface receptors by their agonists, coupled to their second messengers. Exocytosis of granules requires Ca^{2+} mobilisation and Protein Kinase C (PKC) activation. Therefore many platelet receptors on the platelet surface are capable of inducing granule secretion through $G_{\alpha q}$ signalling via PLC-β activation, G_{13} stimulation of RhoA, Gi signalling through PI3K activation, TLR2 activation through PI3K activation and GPVI through Ca^{2+} mobilisation. (Hardy et al., 2004; Woulfe, 2005; Gachet, 2006; Brass, 2010; Cohen et al., 2011; Amison et al., 2012). PKC is one of the most important proteins involved in granular secretion, where the PKCθ isoform is particularly important. PKCθ activation downstream of $G_{\alpha q}$ coupled receptors triggers the phosphorylation of syntaxin-4, a key component of the exocytotic machinery initiating the exocytosis of platelet granules via the SNARE hypothesis. This involves the soluble NSF attachment protein receptor (SNARE), a process by which vesicles relocate and fuse with the extracellular membrane allowing the release of their contents through coiled coil interactions of both vesicle membrane proteins, vSNARES (Synaptobrevin and synaptotagmin) and membrane associated proteins, t-SNARES (Syntaxin 1A, Soluble NSF Attachment Protein-25 (SNAP-25)) (Cattaneo, 2002; Hoylaerts, 2002; Cohen et al., 2011). Stimulation of Ca^{2+} mobilisation also stimulates granular secretion through the promotion of PKC activity.

Secretion of granular content contributes to activation through potentiation of platelet activation and reinforcing aggregation in haemostasis. Importantly, stimulation of granule secretion promotes an increase in mediators of platelet function in the vicinity of unactivated platelets propagating the process of platelet activation.

1.4 A Possible Dichotomy in Platelet Function

1.4.1 The Role of Platelets in Thrombosis and Haemostasis

One of the most extensively described roles of platelets is the response to vascular injury. In their resting state, the platelets discoid shape is maintained by a highly specialised cytoskeleton of spectrin, actin and microtubules (White, 2002; Richardson et al., 2005). The traditional haemostatic response can be characterised by a 3 stage response: initiation, extension, and stabilisation (Brass, 2010). During the initiation phase, platelets are activated by collagen fibrils and thrombin release as a result of endothelial wall damage during vascular injury. These fibrils form a complex with von Willebrand factor (vWF) which in turn interacts with exposed glycoprotein GPIbα on the surface of platelets circulating in the periphery of the blood vessel (Tsuji et al., 1997). These interactions mediated by vWF are promoted by exposure of the vWF A1 domain by high shear which promotes further interactions with GPIbα (Brass, 2010). The interaction of platelets and collagen fibres mediated by vWF slows platelet progression along the vessel long enough to induce platelet activation, triggering inside-out activation of the integrin $\alpha_{IIb}\beta_{3.}$ Additionally, platelet agonists are secreted (ADP, thrombin, TxA_2) into the local area coupled with increased adhesion molecule expression (i.e. P-selectin, PSGL-1) creating a monolayer of platelets over the damaged endothelium (Li et al., 2010). During the second extension phase, circulating platelets come into contact with the activated platelet monolayer resulting in further release of platelet agonists (Li et al., 2010). The increased concentration of platelet agonists in the local region triggers activation of integrin $\alpha_{IIb}\beta_{3.}$ $\alpha_{IIb}\beta_3$ is expressed at high densities on the platelets surface and is exposed to high μM concentrations of fibrinogen within the

vasculature. However under resting conditions the receptor does not become even minimally occupied by fibrinogen; only after Ca^{2+} dependent inside-out signalling is $\alpha_{IIb}\beta_3$ converted from the low affinity to high affinity binding state for fibrinogen (Li et al., 2010). The coagulation cascade then leads to further interactions between fibrin and fibrinogen, fibrin formation is induced following exposure of subendothelial tissue factor to plasma factor VII. Platelet-platelet and platelet-endothelium interactions are increased through interactions between fibrinogen/fibrin or vWF with $\alpha_{IIb}\beta_3$, GPVI or GPIb-IX-V, increasing platelet adherence to the damaged endothelium recruiting additional platelets thus extending the platelet plug. Platelet phospholipids also support factor Xase activity (a complex of FIXa, platelet bound FVIIIa and Ca^{2+}), whilst prothrombinase complex formation is mediated by FXa, platelet bound FVa and Ca^{2+} leading to further thrombin generation. These additional pro-coagulant and clotting factors further stabilise the platelet plug.

The third phase of stabilisation is described by the formation of additional close contacts between platelets within the platelet plug which is embedded with a cross-linked fibrin mesh network. This is capable of withstanding the high shear forces generated by blood flow within the arterial circulation which further enhances the stabilisation of the platelet plug (Brass, 2010). Finally induction of outside-in signalling by $\alpha_{IIb}\beta_3$, $\alpha_{IIb}\beta_3$ triggers contraction of the actin cytoskeleton leading to clot retraction which further helps to stabilise the platelet plug and prevent premature de-aggregation (Brass, 2010).

1.4.2 Platelet Involvement in Inflammatory Disorders

The role of platelets in haemostasis and thrombosis has been well established. However, evidence now exists implicating the platelet as a cell type which can play important roles in inflammatory diseases including allergic inflammation, COPD, inflammatory bowel disease (IBD), atherosclerosis, rheumatoid arthritis, inflammation associated with cardiovascular diseases and the immune response to bacterial infections (Gresele et al., 1993; Moritani et al., 1998; Kowal et al., 2006; Pitchford, 2007b; Boilard et al., 2010; Semple and Freedman, 2010).

Through the use of experimental models, platelets are thought to contribute to inflammatory disorders via numerous mechanisms. These include interactions with inflammatory leukocytes 'priming' them for migration into inflamed tissue where they can contribute to tissue damage and remodelling, and through the ability of platelets to provide a 'bridge' between the innate and the adaptive immune response. Interestingly, the direct migration of platelets into inflamed tissue might lead to a situation where release of pro-inflammatory mediators from platelet granules can contribute to tissue damage and impaired function. In this section the ability of platelets to modulate allergic inflammatory disorders will be discussed.

1.4.2.1 Evidence of Platelet Activation in Allergic Inflammation

Markers of platelet activation have been detected in patients with asthma following allergen provocation, and include elevated platelet derived RANTES, PF-4, β-TG and P-selectin (Moritani et al., 1998; Sullivan et al., 2000; Pitchford et al., 2003a; Kowal et al., 2006). Patients with asthma demonstrate altered platelet function *ex vivo,* because of higher states of platelet activation *in vivo*. For example, platelets

taken from venous peripheral blood of patients with asthma during the allergy season respond poorly to a number of platelet activators (ADP, collagen, thrombin and adrenaline), as demonstrated by reduced secondary aggregation *in vitro*. This was found to correlate with elevated serum immunoglobulin (IgE) levels (> 250U/ml) (Gallagher et al., 1978). This state of 'platelet exhaustion' is believed to be a result of platelet activation by inflammatory stimuli *in vivo* inducing release and subsequent depletion of platelet granular contents. This results in an inability of the platelet to aggregate in response to secondary stimuli *ex vivo* (Maccia et al., 1977; Gallagher et al., 1978; Gresele et al., 1993). Furthermore, it was shown that platelet function recovered in the experiments conducted outside of the allergy (pollen) season (Gallagher et al., 1978). This platelet activation *in vivo* may lead to the apparent mild haemostatic defects that some patients with asthma exhibit, as demonstrated by increased bleeding times (Szczeklik et al., 1986; Ind, 1991).

Alterations in the lifespan of platelets have also been demonstrated. Healthy individuals typically show a platelet lifespan of around 8.9 days. However platelets from atopic asthmatic patients have a significantly decreased lifespan of only 4.7 days, possibly as a result of chronic activation inducing a state of continual platelet consumption. This process could be reversed following corticosteroid treatment of these patients with asthma (Taytard et al., 1986). However, this observation has not been confirmed, as additional groups have failed to demonstrate any difference in the lifespan of platelets between healthy individuals and atopic asthmatic patients (Ind et al., 1985). Patients with asthma also demonstrate a mild thrombocytopenia (10-25%) observed within minutes following allergen exposure in patients with asthma (Sullivan et al., 2000; Kowal et al., 2006), potentially as a result of localised platelet recruitment to sites of inflammation.

Inhibition of platelet derived bronchoactive mediator release (e.g. PAF) abolishes acute AHR in a baboon model of PAF induced bronchoconstriction (Arnoux et al., 1988). Furthermore, platelets have been detected within the lung tissue and bronchoalveolar (BAL) lavage fluid of patients with asthma (Jeffery PK et al., 1989). Interestingly in addition to allergic inflammation, platelet migration towards sites of inflammation has also been detected in other inflammatory diseases including in the synovial fluid with rheumatoid arthritis patients and in the tissue of patients experiencing long periods of ischaemia (Boilard et al., 2010). Thus, the phenomenon of platelet migration and presence in extravascular sites suggests that they may interact directly with resident structural cells and modulate their function.

Release of pro-inflammatory mediators such as β-TG, PAF, PF-4, RANTES, as well as increased surface expression of the adhesion molecule P-selectin are all elevated post allergen challenge in patients with asthma (Gresele et al., 1993; Moritani et al., 1998; Tutluoglu, 2005; Kowal et al., 2006) providing evidence of platelet activation. PF-4 has been shown to stimulate histamine release from basophils, increase IgE receptor expression, induce bronchoconstriction, and provide chemotactic activity and subsequent activation of eosinophils, all of which propagate the inflammatory response (Coyle *et al.*, 1996). Chemokines secreted by platelets, i.e. RANTES, TARC and MDC demonstrate chemotactic properties for inflammatory leukocytes including neutrophils, eosinophils and T cells contributing to the influx of inflammatory cells to the pulmonary tissue following allergen provocation (Lukacs et al., 1996; Lampinen et al., 1999; Sekiya et al., 2000) .

In addition to the previously mentioned mediators released by platelets, 5-HT is also released from platelets. Platelets contain high levels of 5-HT, which is taken up from the plasma via 5-HT transporters (SERTs) and released from dense granules upon

platelet activation (Toh, 1956; Yoshida et al., 2002; Ni and Watts, 2006). Outside of the CNS, 5-HT is synthesised through the catalysation of L-tryptophan to 5-HT by tryptophan hydroxylase 1 (TPH1) (Dürk et al., 2013). 5-HT has been best characterised as a neurotransmitter involved in the regulation of physiologic functions including sleep and mood, but more recently it has emerged as an important inflammatory mediator in the peripheral nervous system. In asthma, elevated levels of plasma 5-HT have been detected, with plasma levels of 5-HT correlating with disease severity and pulmonary function (Malmgren et al., 1982; Lechin et al., 1996). Blockade of the 5-HT transporter through the use of pharmacological antagonists or 5-HT receptor blockade attenuate the development of allergic inflammation and airway wall remodelling in murine models of allergic inflammation (De Bie et al., 1998; Lechin et al., 1998; Lima et al., 2007). More recently, 5-HT synthesised by TPH1 has been shown to be critical in the development of allergic airway inflammation with significantly elevated levels of 5-HT present in BAL fluid (Dürk et al., 2013). More importantly however, this study showed that platelet-derived and not mast-cell derived 5-HT was important in leukocyte infiltration and the development of BHR (Dürk et al., 2013), suggesting platelet derived 5-HT release as a potential future therapeutic target in the treatment of allergic inflammation.

One of the key processes involved in leukocyte recruitment in inflammation is the formation of platelet-leukocyte conjugates, a process known to be P-selectin-PSGL-1 dependent (Moritani et al., 1998; Hayward et al., 1999; Pitchford et al., 2005; Zarbock et al., 2006; Kornerup et al., 2010). Platelet P-selectin expression levels and circulating platelet-leukocyte conjugates are significantly elevated in asthmatic patients following allergen provocation as well as the frequency of conjugate

formation (Gresele et al., 1993; Pitchford et al., 2003b, 2005; Kowal et al., 2006; Johansson et al., 2012). This function of platelets in allergic inflammation is of particular interest as leukocytes bound to platelets demonstrate increased integrin activation on the surface (Johansson et al., 2012; Johansson and Mosher, 2013; Johansson, 2014). Indeed, P-selectin interactions of platelets with blood eosinophils results in subjects with non-severe asthma expressing α4β1 and αMβ2 present in an intermediate-activity state. Eosinophils in this state are said to be pre-activated 'primed' eosinophils ready to facilitate adhesion onto the endothelium (figure 1.4) (Johansson and Mosher, 2013). This has been confirmed with *in vitro* studies where platelet-induced adhesion of eosinophils to endothelium is enhanced with platelets isolated from asthmatic subjects when compared to platelets taken from healthy controls (Ulfman et al., 2003). These 'primed' eosinophils bound with platelets via P-selectin-PSGL-1 interactions provide a link between platelet activation and eosinophilia in nasal secretions of infant and adolescent asthmatic patients (Benton et al., 2010).

Evidently, the involvement of platelets in allergic inflammation adds to an already complex web of cellular interactions involved in propagating the allergic inflammatory response. In the next section, the different ways by which platelet signalling drives the inflammatory responses will be investigated in further detail.

1.4.2.2 Platelet Pulmonary Recruitment

Platelets have been observed to undergo diapedisis in sections of lung from asthmatic patients as well as from lung samples obtained from mice, rabbits and guinea pigs sensitised and challenged to allergen (Jeffery PK et al., 1989; Pitchford et al., 2008). Indeed in allergic inflammation, a mild systemic thrombocytopenia (10-25%) has been observed in asthmatic patients following allergen challenge (Maestrelli et al., 1990; Sullivan et al., 2000; Kowal et al., 2006). This migration of platelets towards allergen has also been confirmed *in vitro* and has been shown to be dependent on allergen/allergen specific IgE interactions with the FcεRI receptor (Pitchford et al., 2008). Further research has begun to show that in addition to asthma, platelet migration has also been seen to occur in other inflammatory diseases; including accumulation within the synovial fluid of joints in rheumatoid arthritis (Boilard et al., 2010), and recruitment to the vascular wall following periods of ischaemia (Kraemer et al., 2010). Platelets have been shown to express functional chemokine receptors (CCR1, CCR3, CCR4 and CXCR4) (Clemetson et al., 2000), and ligands of these receptors including SDF1α (CXCL4), MDC (CCL-17), and TARC (CCL-22) have all shown the capacity to activate platelets (Abi-Younes et al., 2000; Kowalska et al., 2000). Indeed, platelets have previously been shown to migrate towards SDF-1α and f-MLP *in vitro* demonstrating that platelets are indeed motile cells (Czapiga et al., 2005; Kraemer et al., 2010). It is therefore possible that platelet migration is modulated by these chemokines. Indeed, evidence suggests that platelets can be recruited to the lungs immediately following allergen exposure in a murine model of ovalbumin induced pulmonary inflammation (Yoshida et al., 2002; Pitchford et al., 2008), demonstrating that allergen/chemokines may be capable of inducing platelet activation itself.

1.4.2.3 Platelet Involvement in Chronic Inflammation

Patients with chronic asthma demonstrate substantial remodelling of the pulmonary architecture, with thickening of the airway epithelium and smooth muscle combined with increased reticular-fibre deposition within the extracellular matrix resulting in impaired lung function. In animal models, these characteristics can be mimicked through chronic exposure to aerosolised allergen, and in platelet depleted mice, these remodelling processes observed following chronic allergen exposure are significantly reduced (Pitchford et al., 2004). This implies that platelets contribute to wound repair through the release of cellular mitogens such as insulin-like growth factor (IFG), transforming growth factor β (TGFβ), vascular endothelial growth factor (VEGF), epidermal growth factor (EGF) and platelet derived growth factor (PDGF).

As platelets appear to possess the ability to migrate into inflammed tissues, a direct mechanism by which platelets can promote the inflammatory process is possible. In addition to chemokines and platelet agonists, platelet granules also contain numerous degradative enzymes including the matrix metalloproteinase's (MMP1, MMP2, MMP3, MMP14 and potentially MMP9) (Seizer and May, 2013). Their main function is the degradation of the extracellular matrix. In atherosclerosis this leads to destabilisation of the thrombi and the eventual rupturing of the atherosclerotic plaque with vascular wall rupture being the main cause of mortality in atherosclerotic patients (Agewall, 2006).

Additionally, platelets from patients with asthma also release arachidonic acid metabolites and phospholipid metabolites (Plaza et al., 1995). Hydroxyaicosatetraenoic acid (12-HETE) production is stimulated by the release of

the platelet specific enzyme 12-lipoxygenase. 12-HETE also demonstrates strong chemotactic activity for eosinophils (Marcus et al., 1984), and is taken up by neutrophils for the production of 20-diHETE. Neutrophils alone are incapable of producing 20-diHETE and require the uptake of platelet produced 12-HETE for its production (Marcus et al., 1984, 1987, 1988). 20-diHETE has chemoattractant properties for neutrophils but also stimulates the production of leukocyte 5-lipoxygenase involved in the production of leukotrienes (LTC_4, LTD_4, LTE_4 and LTB_4) (Maclouf and Murphy, 1988). Leukotrienes are a potent family of inflammatory mediators capable of inducing bronchospasm, increased vascular permeability, mucus hyper secretion (Maclouf and Murphy, 1988), induction of AHR (Diamant et al., 1997) and inflammatory cell recruitment. Whilst the cysteinyl leukotrienes (LTC_4 and LTD_4) specifically induce eosinophil recruitment, LTB_4 demonstrates non-specific chemoattractant properties for leukocytes. Interestingly, platelets and neutrophils are capable of synergism in the metabolism of arachidonic acid, as leukocyte derived LTA_4 can be converted to LTC_4 by platelets through gluthathion-s-transferase (Maclouf and Murphy, 1988). However this requires direct contact between leukocytes and platelets through platelet p-selectin and PSGL-1 (Maclouf and Murphy, 1988).

1.4.2.3 Platelet Involvement in Bridging the Innate and Adaptive Immune Responses

Platelets posses many receptors involved in the recognition of antigens including the toll-like receptors (TLRs) which recognise components of pathogens such as lipopolysaccharides (LPS) contained within the bacterial cell wall. Platelets express 3 receptors of the TLR family, TLR2, 4 and 9 (Aslam et al., 2006; Semple and

Freedman, 2010). Furthermore, platelets also express both the high (10^{-7} M) and low (10^{-9} M) affinity IgE receptors (FcεRI and FcεRII respectively) as well as IgG receptors (FcγRI, FcγRII) (Joseph et al., 1986, 1997). The first indication of IgE induced activation of platelets was observed by Benveniste in 1972, where leukocyte dependent histamine release was observed from rabbit platelets. Later identification of immunoglobulin receptors on platelets strongly implicated a potential role of platelets within the adaptive immune response. Platelet activation induced by stimulation of FcεRI can induce platelet release of 5-HT and RANTES, suggesting that platelet stimulation by IgE might affect the allergic inflammatory response (Hasegawa et al., 1999), given that similar cross-linking of allergen specific IgE with the FcεRI receptor on mast cells results in mast cell degranulation and the release of both preformed and newly synthesised mediators including ROS, eicosanoids, 5-HT, and histamine (Hogan and Schwartz, 1997). Importantly, in normal individuals, IgE binds approximately 20-30% circulating platelets, whilst in asthmatic patients, this binding increases to approximately 50% (Maccia et al., 1977; Joseph et al., 1986), a phenomenon also observed in circulating platelets obtained from allergen-sensitised mice (Pitchford et al., 2008). Furthermore platelets collected from atopic individuals were shown to have significantly elevated IgE stored within the platelet granules which correlated with increased serum IgE (Gallagher et al., 1978). In agreement with this, allergen-sensitised mice demonstrate increased platelet bound-IgE (Pitchford et al., 2008).

The implication of platelets in bridging the innate and adaptive immune responses can be seen in patients of immune thrombocytopenia (ITP), a condition characterised by both accelerated clearance of autoantibody-sensitised platelets and suboptimal platelet production. Studies have demonstrated that patients with ITP suffer from

disturbances to both the innate and adaptive immune responses (reviewed in Lazarus 2013). Other studies have implicated platelets in a regulatory role for the maintenance of Th cell homeostasis, where platelet derived PF4 has been implicated in limiting Th17 cell differentiation (Shi et al., 2014).

One of the key components of platelet activation and their contribution to immunity and inflammation lies in the interactions of granule released CD40 and CD40L present on activated platelets. CD40 is a phosphorylated membrane glycoprotein constitutively expressed on platelets (Inwald et al., 2003) which interact with leukocytes expressing CD40 ligand (CD40L) (Vanichakarn et al., 2008). Whilst the role of CD40 and CD40L has been extensively researched in atherosclerosis, it's role in inflammation is less well defined. Recent work has identified that vast numbers of CD40+ and CD40L+ cells are detectable in conditions of chronic inflammation and autoimmune disorders (Danese et al., 2004). Engagement of platelet CD40 with CD40L has been shown to induce platelet activation demonstrated by increased P-selectin expression (Danese et al., 2004). Furthermore, activation induced through CD40 and T cell derived CD40L interactions triggered granule release of the leukocyte chemokine RANTES, enhancing T cell recruitment through endothelial cell activation. This produces an amplification loop of T cell recruitment mediated by platelet and T cell interactions (Henn et al., 1998; Danese et al., 2004). These key interactions between CD40 and CD40L of platelets and leukocytes potentially link the provocation of tissues with the adaptive immune response. Platelet activation by thrombin has been shown to increase levels of platelet CD40L inducing the maturation of dendritic cells as indicated by elevated surface expression of both CD8- and CD83 on dendritic cells and increased levels of IL-12 production (a process dependent on platelet-derived CD40L) (Czapiga et al., 2004). Platelet

induced maturation of dendritic cells potentiates co-stimulatory signals from dendritic cells inducing T cell activation and survival (Czapiga et al., 2004). This suggests that platelet signalling can lead to the maturation of APCs and modulate their subsequent effects on the adaptive immune response.

1.4.2.4 Platelet Involvement in Inflammatory Cell Recruitment

A large amount of research has recently described the ability of platelets to interact with inflammatory leukocytes within the circulation, with platelet-leukocyte conjugates significantly elevated in asthma (Gresele et al., 1993; Pitchford et al., 2003a, 2005; Kowal et al., 2006; Johansson et al., 2012). Other inflammatory disorders are also associated with heightened platelet activation including COPD, atherosclerosis and rheumatoid arthritis (Ott I, et al., 1996; Neumann et al., 1997; Joseph et al., 2001; Sarma, 2002; Huo et al., 2003; Pitchford et al., 2003b, 2005; Irving et al., 2008). As P-selectin bound leukocytes and the resulting contact-dependent activation of leukocytes have been shown to induce the conversion of the αMβ2 and α4β1 integrins to the intermediary activity-state (Johansson and Mosher, 2013), leading to increased adhesion of leukocytes to the vascular endothelium via P-selectin-dependent adhesion (Jawieñ et al., 2002; Ulfman et al., 2003), it suggests a mechanism by which leukocytes can become activated and subsequently adhere to damaged endothelial cells prior to diapedisis into the tissue.

The interaction of platelet derived P-selectin with its counter ligand PSGL-1 on the surface of leukocytes leads to leukocyte activation (Pitchford et al., 2005; Kornerup et al., 2010; Johansson et al., 2012; Page and Pitchford, 2013). The formation of platelet-leukocyte conjugates seen in the clinic has been replicated in a number of

animal models (Sanctis et al., 1997; Broide et al., 1998; Lukacs et al., 2002; Pitchford et al., 2003a, 2005) with these platelet-leukocyte aggregates forming as a result of adhesion interactions without the induction of platelet aggregation (Figure 1.3).

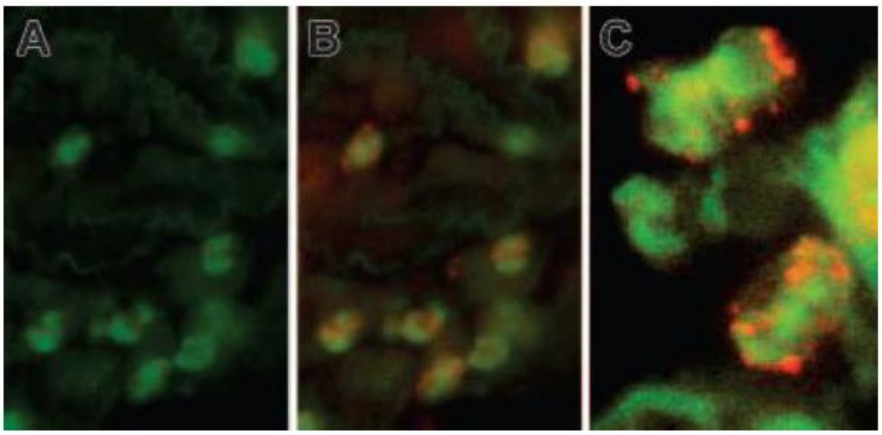


Figure 1. 4. Identification of platelet-eosinophil complexes taken from allergen-sensitised mice after allergen exposure

Lung samples taken from allergen challenged mice were stained with rat anti-MBP for eosinophils (green fluorescence) and goat anti-CD41 for platelets (red fluorescence). **A** Image with green fluorescence filter only **B** Red and green fluorescence filters combined, showing eosinophils complexed with platelets **C** Individual platelets (red) attached to eosinophils (green). (Pitchford et al., 2005)

P-selectin is an integral membrane glycoprotein expressed by platelets. In resting conditions, P-selectin is stored within the α-granules of the platelet. Cellular activation induces rapid relocation of P-selectin from the secretory α-granules to the plasma membrane of the platelet (Gachet, 2006). Here, P-selectin recognises its counter ligand; PSGL-1 expressed on the surface of polymorphonuclear (PMN) cells, initiating the reversible interaction of platelets and leukocytes increasing circulating platelet-leukocyte aggregate numbers (Joseph et al., 2001; Jawieñ et al., 2002; Pitchford et al., 2003a, 2005; Zarbock et al., 2006; Irving et al., 2008; Kornerup et al., 2010) . These interactions then support contact-dependent activation

of leukocytes and subsequent intergin expression, facilitating stable interactions between the 2 cell types. Platelet PSGL-1 expression then allows the tethering and subsequent rolling of platelet-leukocyte conjugates to endothelium through interactions with endothelial derived selectins. This rolling subsequently progresses to firm adhesion through integrin interactions via inside-out signalling from either platelets or leukocytes with the endothelium (Johansson et al., 2012; Johansson and Mosher, 2013) (figure 1.5).

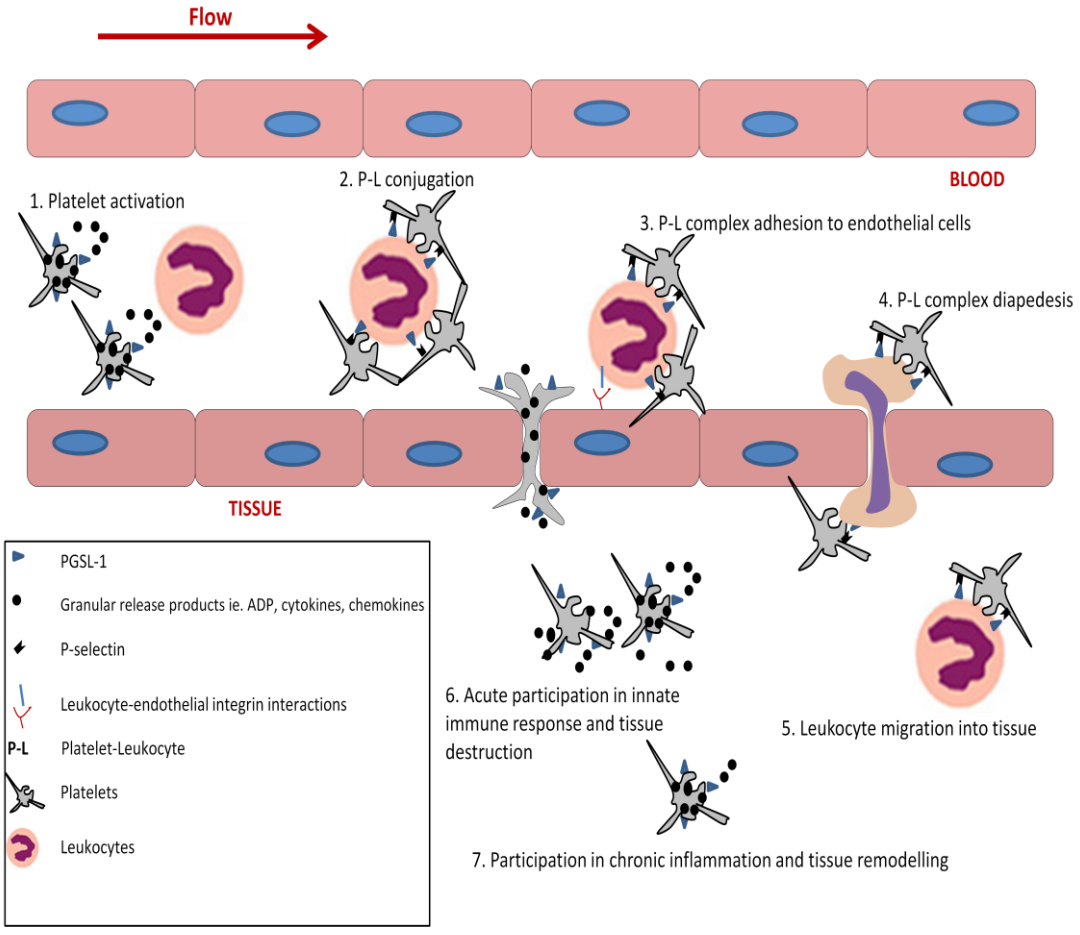


Figure 1. 5. Contribution of platelets to inflammatory processes

Platelet activation results in granular secretion of inflammatory mediators and upregulation of adhesion molecules. Expression of P-selectin and PSGL-1 triggers platelet-leukocyte conjugation eventually targeting the P-L complex to the endothelium for firm adhesion through integrin activation. This allows the P-L complex to migrate across the endothelium into damaged tissue (Amison et al., 2012)

The requirement of platelets for leukocyte recruitment has been confirmed in a variety of *in vivo* animal studies, in particular models of allergic inflammation, allergen induced eosinophil and lymphocyte recruitment is abolished in platelet depleted rabbits, guinea-pigs and mice (Lellouch-Tubiana et al., 1988; Coyle et al., 1990; Pitchford et al., 2003a, 2005). This allergen induced eosinophilia was then restored following platelet reinfusion thus confirming the role of platelets (Pitchford et al., 2003b). *Pitchford et al* 2005, expanded on this through reinfusion of thrombocytopenic mice with platelets treated with the P-selectin blocking antibody RB40.34. When platelets treated with the anti-P-selectin antibody were reinfused, allergen induced eosinophilia remained suppressed (Broide et al., 1998; Lukacs et al., 2002; Pitchford et al., 2003b, 2005). This clear involvement of P-selectin in inflammatory cell recruitment has led to possible targeting of the P-selectin/PSGL-1 interactions as a therapeutic target for inflammatory cell recruitment. The pan-selectin antagonist Bimosiamose (TBC1269) can significantly reduce leukocyte recruitment in a variety of *in vivo* assays including significant attenuation of neutrophil accumulation at infarction sites in a model of myocardial infarction/reperfusion injury (Onai et al., 2003) as well as attenuation of late asthmatic responses following allergen challenge (Beeh et al., 2006).

It is important to consider that whilst P-selectin and PSGL-1 interactions are clearly important for leukocyte adhesion and transmigration, they are not the only adhesion molecules involved, and there is probably both a sequential and time dependent interplay of molecules expressed on the platelets surface as well as the release of soluble mediators. For example, platelets have previously been reported to secrete soluble CD40L (sCD40L) upon activation as well as up-regulating membrane expression of CD40L (Henn et al., 1998; Vanichakarn et al., 2008; Chandler et al.,

2010). Vanicharkan *et al.* 2008 have reported that platelet derived CD40L can interact with CD40 expressed on neutrophils stimulating the release of ROS capable of activating platelets and enhancing sCD40L release through a positive feedback mechanism (Vanichakarn et al., 2008) propagating the inflammatory response. Additionally, the increased expression of CD40L on platelets enables interactions with endothelial cells expressing CD40 triggering production of pro-inflammatory cytokines and the upregulation of adhesion molecules (Henn, 2001; Danese et al., 2011). This elevation in adhesion molecule expression generates increased signals involved in the recruitment and extravastion of leukocytes at the site of inflammation (Henn et al., 1998; Danese et al., 2004). Indeed, stimulation of endothelial cells by platelet derived CD40L has been shown to significantly enhance inflammatory cell recruitment in atherosclerosis (Büchner et al., 2003). Platelet also express CD40 on their surface, which allows for interactions with platelets expressing CD40L increasing the formation of platelet aggregates (Inwald et al., 2003), but also up-regulated surface expression of platelet P-selectin, increased α- and dense-granule secretion, enhanced platelet-leukocyte adhesion and platelet integrin activation (Inwald et al., 2003), all of which continue to propagate the inflammatory response.

Platelets also express intercellular adhesion molecule-2 (ICAM-2) (Diacovo et al., 1994) which is capable of mediating leukocyte-platelet interactions via CD11a/CD18 (Leukocyte function associated antigen-1, LFA-1) and firm adhesion following selectin mediated tethering (Kuijper et al., 1998). Additionally, platelet endothelial interactions can be mediated by platelet expressed platelet endothelial cell adhesion molecule 1 (PECAM-1) and junctional adhesion molecule 1-3 (JAM 1-3) (Chavakis et al., 2004). Indeed, antagonism of these interactions has been reported to

significantly attenuate neutrophil emigration into inflamed tissue in a murine model of peritonitis (Chavakis et al., 2004).

This demonstrates that whilst platelet-leukocyte interactions via P-selectin-PSGL-1 interactions are important in the formation of platelet-leukocyte conjugation and subsequent contact-dependent activation of the leukocytes, additional adhesion molecules are upregulated on the platelet surface upon activation. These contribute to further platelet, leukocyte and endothelial cell activation propagating the inflammatory response through the production of pro-inflammatory mediators as well as enhancing inflammatory cell recruitment to sites of inflammation.

1.6.1 Platelet Purinergic Receptors

The Purines ATP and ADP have previously been implicated in parameters linked to inflammatory processes. Stimulation of platelets with ADP induces significant increases in P-selectin expression**.** Furthermore, antagonism of both the $P2Y_1$ and $P2Y_{12}$ receptors have previously been shown to inhibit P-selectin expression and the formation of these platelet-leukocyte conjugates within the circulation (Klinkhardt et al., 2002; Storey et al., 2002; Leon et al., 2003; Evangelista et al., 2005; Perneby et al., 2007). The ability of platelet purinergic receptors to modulate platelet function that is relevant to both haemostasis (platelet shape change, aggregation, integrin $\alpha_{IIb}\beta_3$ activation, thrombus stabilisation) and inflammation (P-selectin expression, Platelet-leukocyte conjugation) demonstrate possibilities by which purine stimulation of platelets may contribute to their role in inflammation. Whilst the effects of purine stimulation of the haemostatic functions of platelets are well

documented, the role of the purines in the inflammatory setting of platelets clearly requires more detailed analysis.

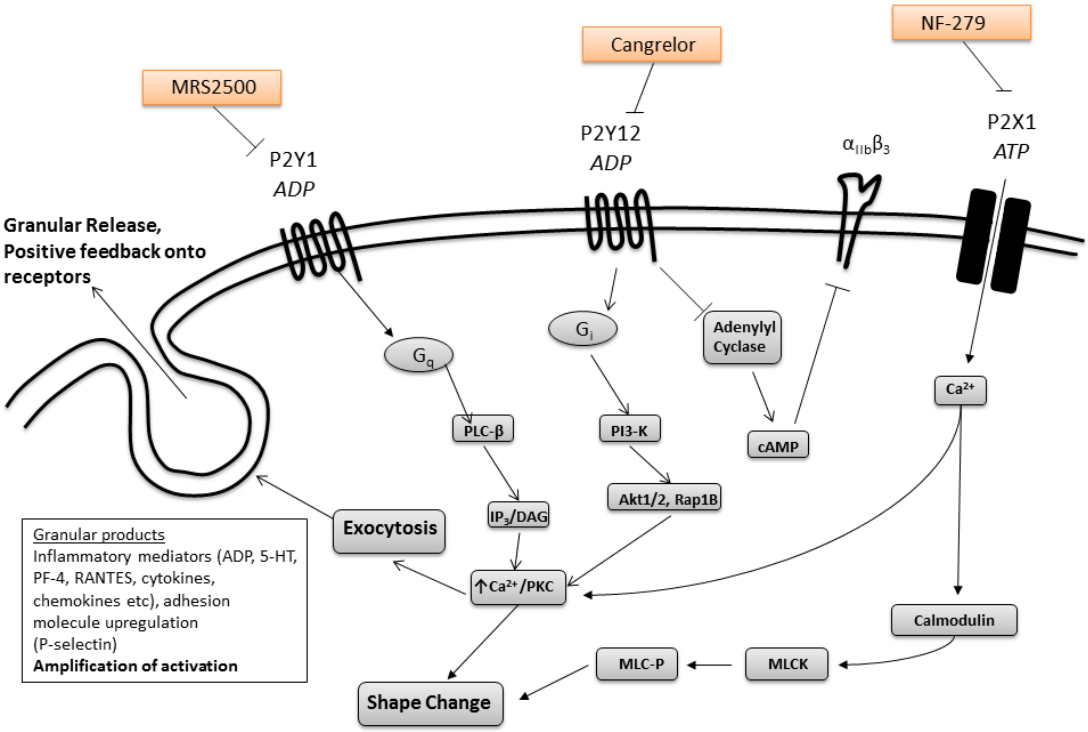


Figure 1. 6. Involvement of platelet receptors in the inflammatory response

The platelet surface express a family of receptors activated by the purines, ADP or ATP, and have long been known to be capable of inducing platelet activation resulting in aggregation, granule secretion, adhesion molecule upregulation and interactions with inflammatory cells (Fabre et al., 1999; Léon et al., 1999; Storey et al., 2000; Eckly et al., 2001; Hechler et al., 2003; Leon et al., 2003, 2004; Cattaneo et al., 2004). Whilst the family of Purinergic receptors is extensive, only 3 receptor subtypes are expressed on the platelets surface, two from the GPCR P2Y family of receptors $P2Y_1$ and $P2Y_{12}$ and one from the ionotropic P2X family, $P2X_1$ (Figure 1.6). The $P2X_1$ receptor is a ligand gated cation ion channel activated by endogenous

ATP resulting in a rapid influx of Ca^{2+} ions, whilst both the $P2Y_1$ and $P2Y_{12}$ receptors are stimulated by endogenous ADP initiating separate signalling cascades controlled by their respective linked G Proteins (Woulfe, 2005; Gachet, 2006, 2008; Mahaut-Smith et al., 2011) (Figure 1.6).

During normal haemostasis, endothelial cells express the enzyme NTPDase1 (also known as CD39) which catalyses the breakdown of ATP to ADP and subsequently into inactive AMP, thus eliminating 2 major molecules resulting in the interaction of endothelial cells and blood platelets (Marcus et al., 2005). However, following vascular injury, endothelial cells become damaged exposing the sub endothelial matrix, whilst also decreasing the elimination of ATP and ADP through decreases in NTPDase1 availability, triggering platelet activation through purinergic stimulation (Marcus et al., 2005).

1.6.2 Platelet $P2X_1$ Receptor Signalling

To date, 7 different subunits of the P2X receptor family have been identified, $P2X_{1-7}$. Each of the different subunits possess the same topology which consists of 2 transmembrane spanning domains (TMD1 and TMD2), a large extracellular loop domain and intracellular C and N termini. Under normal conditions a functional receptor exists as either homotrimers or heterotrimers varying in kinetics and pharmacology, however all P2X receptor subtypes are activated by ATP (Ralevic and Burnstock, 1998; North, 2002; Burnstock, 2007; Surprenant and North, 2009; Hu and Hoylaerts, 2010). Whilst P2X receptors do not appear to have common consensus ATP binding sequences it is suspected that ATP molecules interact with the extracellular loop between TMD1 and TMD2 enabling up to 3 molecules of ATP to bind to a single functional receptor (Roberts and Evans, 2007). The $P2X_1$ receptor

expressed in platelets, following stimulation by ATP increases cellular permeability to Ca^{2+} ions (although small levels of Na^{2+} ion permeability have also been observed) (Sun et al., 1998) providing a substantial source of direct Ca^{2+} influx into the platelet post activation. This rapid influx of Ca^{2+} can instigate a number of reactions including depolarisation of the platelet membrane to enhance signalling through the G_q coupled receptors including the $P2Y_1$ receptor (Toth-Zsamboki et al., 2003). Rapid influx of Ca^{2+} ions induced by ATP receptor stimulation of the $P2X_1$ receptor can trigger a rapid but reversible shape change peaking within seconds, and low level inside-out activation of integrin $\alpha_{IIb}\beta_3$ through either calmodulin (CaM) dependent activation of myosin light chain kinase (MLCK) or PKC activation (Toth-Zsamboki et al., 2003) (Figure 1.6). Binding of Ca^{2+} with CaM results in a conformational change of CaM, inducing its dimerisation and remodelling of its active sites (Toth-Zsamboki et al., 2003). This alteration in CaM's structure causes the displacement of its auto-inhibitory domains of numerous target proteins including MLCK (Toth-Zsamboki et al., 2003), elevating levels of myosin light chain phosphorylation (MLCP). Alternatively, $P2X_1$ mediated rises in intracellular Ca^{2+} can trigger PKC activation, which when combined with CaM activation triggers phosphorylation of ERK2 (Toth-Zsamboki et al., 2003; Oury et al., 2006). Both mechanisms increase phosphorylation of myosin light chain kinase resulting in platelet shape change, however ERK2 phosphorylation is much slower than shape change and is thus more likely to be involved in the amplification of aggregation, as demonstrated through impaired aggregation and dense granule secretion at low levels of GPVI stimulation in the presence of a blocker of ERK2 phosphorylation (Oury et al., 2001; Mahaut-Smith et al., 2011). Importantly, stimulation of $P2X_1$ receptors alone appear to be capable only of inducing reversible platelet shape change without

subsequent aggregation (Rolf et al., 2001). This Ca^{2+}/CaM dependent regulation of MLC kinase may be fundamental in the regulation of platelet activation given the importance of MLCK in the regulation of actomyosin reorganisation and subsequent cytoskeleton reorganisation. A process involved in key stages of platelet activation such as shape change and granule secretion (Johnson et al., 2007) (Figure 1.6). Phosphorylation of the myosin regulatory light chain subunit produces an elevation of actin-activated myosin ATPase activity resulting in cross-bridging of both actin and myosin. Cross-bridging of actin and myosin instigates a number of events resulting in the centralisation of secretory granules within the platelet (Ikebes et al., 1994; Toth-Zsamboki et al., 2003). $P2X_1$ regulation of shape change and granule secretion may contribute to positive feedback of additional platelet agonists initiating subsequent platelet aggregation (Gachet, 2008; Mahaut-Smith et al., 2011).

Studying the impact of the $P2X_1$ receptor in platelet activation has proved difficult due to rapid desensitisation of the receptor following stimulation. However desensitisation can be prevented through the hydrolysis of excess ATP using apyrase (Sun et al., 1998; Rolf et al., 2001; Mahaut-Smith et al., 2011). When desensitisation of the $P2X_1$ receptor has been suitably reduced the $P2X_1$ receptor appears be to incapable of inducing platelet aggregation alone, although it has been implicated in collagen and shear induced aggregation (Hechler et al., 2005). Indeed collagen induced aggregation and secretion is decreased in $P2X_1$ deficient platelets and selectively $P2X_1$ desensitised platelets (Hechler et al., 2003). Furthermore, platelet adhesion and thrombus size was decreased in $P2X_1$ deficient mice combined with a decrease in thrombus removal time (Oury et al., 2001; Hechler et al., 2003; Kahner et al., 2006). Stimulation of $P2X_1$ receptors triggers initial platelet shape change and granular centralisation without secretion, potentially helping to amplify platelet

responses mediated by lower concentrations of alternate platelet agonists (Mahaut-Smith et al., 2011). Indeed, it appears that $P2X_1$ receptor signalling is capable of priming G_q coupled receptors i.e. PAR receptors, TP receptors and $P2Y_1$ receptors with significant potentiation of their responses observed in the presence of combined stimulation of the $P2X_1$ receptor (Oury et al., 2001; Erhardt et al., 2006; Gachet, 2006; Kahner et al., 2006). However this potential synergism between $P2X_1$ receptors and alternate G_q coupled receptors requires further investigation as conflicting studies exist for the role of $P2X_1$ receptors in thrombin induced activation (Hechler et al., 2003). Whilst further clarification is required, it demonstrates a potential role for the potentiation of G_q stimulated activation of platelets, providing an important basis by which the platelets response can be amplified to achieve full activation and events including thrombus generation.

The synergy between $P2X_1$ receptors and G_q coupled receptors can be readily seen with the $P2Y_1$ receptor. Whilst the $P2Y_1$ receptor alone can only induce weak secretion of platelet granules, ATP released during these events can significantly enhance intracellular Ca^{2+} elevation and whilst $P2X_1$ receptor blockade has no impact on ADP-induced platelet aggregation, the subsequent activation of the $P2X_1$ receptor accelerates the mobilisation of intracellular Ca^{2+} (Mahaut-Smith et al., 2011) a process which may benefit platelet adhesion to damaged vessel walls which may otherwise fail to adhere if not activated in a small time window (Mahaut-Smith et al., 2011). Thus, specific stimulation of $P2X_1$ receptors alone are capable of inducing platelet activation but only to the level of reversible shape change and granule secretion but are capable of the modulation and potentiation of a number of additional platelet agonists. The $P2X_1$ receptors have the potential to substantially modulate haemostatic functions under a variety of conditions through modulation of

platelet shape change and intracellular Ca^{2+} mobilisation in platelets. Indeed, whilst the role of $P2X_1$ receptor activation in inflammation hasn't been extensively researched, initial studies have begun to implicate it in a number of inflammatory parameters including the promotion of neutrophil migration by ATP induced $P2X_1$ receptor activation on neutrophils (Lecut et al., 2009). Later studies have also implicated a protective role for $P2X_1$ receptor activation in endotoxemia through dampening of neutrophil activation (Lecut et al., 2012). Whilst the emerging area of $P2X_1$ involvement in inflammation currently requires further research, it opens the possibility of a potential role of the $P2X_1$ receptor in platelet mediated inflammatory responses.

1.6.3 Platelet P2Y Subfamily

The second class of purinergic receptor present in platelets are the ADP sensitive metabotropic P2Y receptors. At present this subfamily is accepted to contain 8 different human subtypes: $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$ and $P2Y_{14}$ (table 1.2), with the missing numbers accounted for by either non-mammalian subtypes or receptors with similar sequence homology, but with no apparent functional sensitivity to the nucleotides (Ralevic and Burnstock, 1998; Burnstock, 2007). All of the receptors demonstrate a similar molecular structure typical of the G protein coupled family of receptors (GPCRs) with an extracellular N-terminal domain and a intra-cellular C-terminal domain separated by 7 transmembrane spanning domains (**figure 1.9**) (Deupi and Kobilka, 2007). Activation of the GPCR purinergic receptors occurs along the extracellular loop segment connecting transmembrane domains IV and V (Burnstock, 2007; Deupi and Kobilka, 2007).

When an agonist binds to the receptor a conformational change is triggered within the receptor, through a twisting and rotating movement of transmembrane domain (TMD) VI around the rotomer toggle switch (relative to TMD III) or the release of the ionic lock freeing up the attached G protein for guanine exchange factors (GEFs) allowing the exchange of Guanine di-phosphate (GDP) for Guanine tri-phosphate (GTP). The G Protein itself consists of 3 domains α, β and γ. On activation (the exchange of GDP for GTP) the α subunit dissociates from the β/γ complex where both the α subunit and the β/γ complex can then further affect intracellular signalling proteins depending on the original G Protein (Wess, 1997).

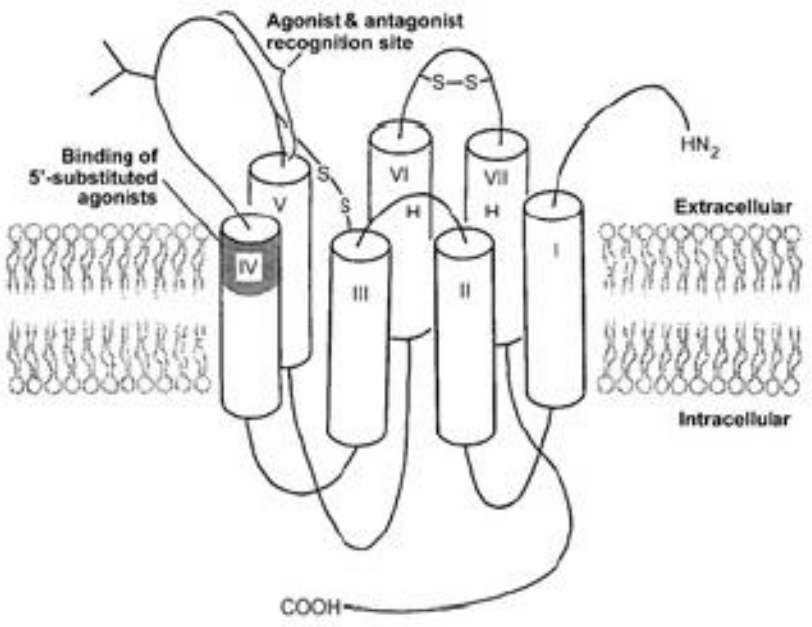


Figure 1. 7. Diagrammatic representation of P2Y receptor structure

(Burnstock, 2007)

The 2 P2Y receptors expressed on the surface of platelets, $P2Y_1$ and $P2Y_{12}$ respond to ADP as their endogenous ligand and synthetic agonists are available for both receptors such as MRS2365 ($P2Y_1$) and 2-methylthio-ADP (2-MeSADP) ($P2Y_{12}$). Whilst ATP activates the $P2X_1$ receptor , it acts as an antagonist to both the $P2Y_1$

and $P2Y_{12}$ receptors (Ingall et al., 1999; Kauffenstein et al., 2004). Extensive research has implicated both the $P2Y_1$ and $P2Y_{12}$ (formally P2T) receptors in the regulation of platelet aggregation since the 1960s (Born and Cross, 1964). In subsequent years, both the $P2Y_1$ and $P2Y_{12}$ receptors have been analysed extensively resulting in the elucidation of their roles in hemostasis.

Receptor	Main Distribution	Transduction Mechanism
$P2Y_1$	Epithelial and endothelial cells, Platelets, Immune Cells, Osteoclasts	G_q/G_{11} PLC-β activation
$P2Y_2$	Epithelial and endothelial cells, Immune Cells, Kidney tubules, Osteoblasts	G_q/G_{11} PLC-β activation, G_i
$P2Y_4$	Endothelial Cells	G_q/G_{11} PLC-β activation, G_i
$P2Y_6$	Eplithelial Cells, Placenta, T Cells, Thymus	G_q/G_{11} PLC-β activation, G_i
$P2Y_{11}$	Spleen, Intestine, Granulocytes	G_q/G_{11} PLC-β activation, G_s
$P2Y_{12}$	Platelets, Glial Cells	$G_{i/o}$ Adenylyl Cyclase Inhibition
$P2Y_{13}$	Spleen, Brain, Lymph nodes, Bone marrow	$G_{i/o}$ Adenylyl Cyclase Inhibition
$P2Y_{14}$	Placenta, Adipose tissue, Stomach, Intestine, Discrete brain regions	G_q/G_{11}

Adapted from Burnstock *et al.* 2007

Table 2. Cellular distribution of the P2Y receptor subtypes

1.6.3.1 Platelet $P2Y_1$ Signalling

The $P2Y_1$ receptor is expressed on the surface of platelets in substantially lower levels compared to other platelet receptors, for example the PAR1 receptor. The PAR1 receptor is expressed in levels ranging between 1000-2000 receptors per platelet, whereas platelets only express between 100-150 $P2Y_1$ receptors per individual platelet (Baurand et al., 2001)

ADP induced activation of platelet $P2Y_1$ receptors triggers activation of the G_q coupled G Protein initiating α subunit dissociation from the β/γ complex allowing phospholipase C beta (PLCβ) activation downstream of the $P2Y_1$ receptor (Gachet, 2006). Once activated, PLCβ mediates the formation of Inositol-3-phosphate (IP_3) and Di-acylglycerol (DAG) from PIP_2 (phosphoinositol-bis-phosphophosphate). Elevation in intracellular IP_3 as a result of PLCβ activation increases IP_3 binding to the IP_3 receptor on the walls of intracellular compartments initiating Ca^{2+} release from internal stores, thus mobilising intracellular Ca^{2+} (Lian et al., 2005). Increases in intracellular Ca^{2+} promote rearrangement of the actin cytoskeleton initiating rapid platelet shape change. In addition to IP_3 formation, PLCβ also results in the production of DAG. DAG triggers Protein Kinase C activation and combined with the elevation of intracellular Ca^{2+} produced through IP_3 production results in the centralisation and secretion of platelet granules (Walker and Watson, 1993). An additional impact of $P2Y_1$ receptor activation is the formation of the potent platelet agonist Thromboxane A_2 (TxA_2) (Samuelsson et al., 1978; Bhagwat et al., 1985; Jin, 2002). DAG also triggers the synthesis of TxA_2 through the synthesis of arachidonic acid (AA). AA is metabolised into endoperoxidases (PGG2 and PGH2) by cycloxygenase (COX), with TxA_2 subsequently being produced from PGH2 by thromboxane synthatase (Samuelsson et al., 1978; Bhagwat et al., 1985; Jin, 2002). TxA_2 is a very unstable agonist with a half life of only around 30-40 seconds (Samuelsson et al., 1978), however despite its short activity it has pronounced biological activity as a platelet agonist inducing aggregation via activation of the platelet TP receptor, amplifying platelet activation.

The importance of $P2Y_1$ signalling in platelet activation has become increasingly clear using a range of $P2Y_1$ specific antagonists including MRS2179, MRS2500 and

through the use of $P2Y_1$ deficient mice. These studies have demonstrated that blockade of $P2Y_1$ signalling induces a severe impairment of platelet activation with defects observed in shape change, Ca^{2+} mobilisation, P-selectin expression, ERK phosphorylation and aggregation (Jin, 1998; Fabre et al., 1999; Léon et al., 1999; Baurand et al., 2001; Leon et al., 2003; Hechler et al., 2006; Garcia et al., 2007). Fabre *et al.* 1999, demonstrated significantly increased bleeding time (~25 minutes) in $P2Y_1$ deficient mice compared with a bleeding time of approximately 10 minutes in wild type mice (Fabre et al., 1999), this was coupled to a failure of $P2Y_1$ deficient mice to increase intracellular Ca^{2+} levels following stimulation with 30μM ADP compared to WT mice or undergo shape change towards 0.3μM (Fabre et al., 1999). Stimulation of $P2Y_1$ deficient mice with either U46619 (TxA_2 analogue) or 1.5u/ml thrombin were capable of inducing normal platelet activation compared to WT mice demonstrating that the inability of ADP to induce platelet activation was a result of a lack of $P2Y_1$ signalling rather than a defect in general platelet activation (Fabre et al., 1999). Other studies have also demonstrated impaired platelet function in $P2Y_1$ deficient mice with transient aggregation to 5μM ADP completely abolished in $P2Y_1$ deficient mice compared to WT mice. Once again however stimulation of $P2Y_1$ deficient mice with 1u/ml thrombin produced normal aggregation compared to WT mice (Léon et al., 1999). These $P2Y_1$ deficient mice have also demonstrated protection against systemic intravascular thrombosis following intravenous injection of ADP or collagen with significant increases in survival rates observed (Fabre et al., 1999; Léon et al., 1999), further demonstrating its critical role in platelet activation. Studies with selective low molecular weight inhibitors such as MRS2500 and MRS2179 have also demonstrated impaired platelet activation and aggregation, with

attenuation of ADP induced aggregation similar to levels observed in $P2Y_1$ deficient mice (Hechler et al., 2006) Baurand et al., 2001).

In addition to impaired Ca^{2+} mobilisation, shape change and platelet aggregation, deficiencies in $P2Y_1$ signalling also results in impaired adhesion molecule expression, demonstrating impaired granule secretion. Stimulation with ADP is known to result in increased P-selectin expression (Storey et al., 2000, 2002; Leon et al., 2003; Evangelista et al., 2005; Perneby et al., 2007). Blockade of the $P2Y_1$ receptor with MRS2500 and MRS2179 can attenuate this ADP-induced elevation in P-selectin expression (Leon et al., 2003, 2004).

Whilst the $P2Y_1$ receptor is clearly involved in platelet activation and hemostasis as demonstrated by its role in platelet activation and increased protection from thrombosis following antagonist treatment, stimulation of the $P2Y_1$ receptor alone is insufficient for full aggregation and can only induce a transient and reversible level of aggregation (Hechler et al., 1998; Baurand et al., 2001). Therefore additional stimulation of the platelets must be involved. More recently, research has begun to implicate the $P2Y_1$ receptor in the RhoA signalling pathway. RhoA is a small GTPase belonging to the family of Rho GTPases consisting of RhoA, Rac and cdc42. Under 'resting' inactive conditions, RhoA is bound to GDP but following activation through linked GPCRs, GDP is switched for GTP through increasing GEF function (Bos et al., 2007).

Rho signalling has previously been implicated with a key role in linking surface receptors to the actin cytoskeleton and in turn, its organisation. Within the vasculature, Rho signalling has been demonstrated to play a regulatory role in a number of different functions, including inflammation, leukocyte migration across

the endothelial barrier, platelet activation, thrombosis and oxidative stress (Eckly et al., 2001; Rolli-Derkinderen et al., 2010; Pleines et al., 2012; Aslan and McCarty, 2013; Ridley, 2013b). Indeed, RhoA has also been implicated in a variety of markers of platelet activation including shape change, filopodia formation, cytoskeletal changes and granule centralisation prior to secretion (Eckly et al., 2001; Pleines et al., 2012). These RhoA mediated events are not followed by subsequent platelet aggregation, this suggests that RhoA mediated events could potentially play a key role in eliciting the inflammatory actions of platelets, whilst demonstrating a redundancy in platelet aggregation.

1.6.3.2 Platelet $P2Y_{12}$ Signalling

The second P2Y receptor subtype present on the surface of platelets is the $P2Y_{12}$ receptor. Whilst the $P2Y_{12}$ receptor is expressed at higher levels than the $P2Y_1$ receptor, with expression levels exceeding 1000 receptors per platelet, its systemic distribution is limited solely to platelets and glial cells (Burnstock, 2007).

The $P2Y_{12}$ receptor is coupled to G_i, with the dissociated α-subunit coupling to and negatively regulating the membrane bound protein adenylyl cyclase (AC). Under resting conditions, AC catalyses the production of cyclic adenosine monophosphate (cAMP), locking the platelet integrin $\alpha_{IIb}\beta_3$ in an inactive conformation preventing or placing a 'brake' on platelet aggregation (Kauffenstein et al., 2001). However, upon $P2Y_{12}$ receptor stimulation and subsequent inhibition of AC, the resulting drop in intracellular cAMP levels removes the block on $\alpha_{IIb}\beta_3$ activation allowing the formation of cohesive interactions between platelets thus initiating platelet aggregation (Kauffenstein et al., 2001; Gachet, 2006; Brass, 2010). Furthermore, activation of the $P2Y_{12}$ stimulates secretion of platelet dense granules which leads to

local generation of thrombin and $TxA_{2,}$ thus amplifying platelet aggregation (Storey et al., 2000)

Given its limited distribution, the $P2Y_{12}$ receptor has been viewed as an attractive therapeutic target for some time, with $P2Y_{12}$ specific thienopyridine compounds such as ticlopidine and clopidogrel being used extensively in antithrombotic therapy (Collaboration, 1994; Barer, 1997; Moussa et al., 1999; Perneby et al., 2007). The identification of specific targeting of $P2Y_{12}$ receptors for anti-platelet therapy began a new wave of development of more potent and specific $P2Y_{12}$ receptor antagonists. Clopidogrel is itself a prodrug, and due to liver enzyme polymorphisms some patients are less able to metabolise clopidogrel into its active form leaving them resistant to Clopidogrel therapy (Gachet, 2006). Therefore, subsequent drug research and development created the third generation of $P2Y_{12}$ receptor antagonists including Prasugrel (LY640315), ticagrelor (AZD6140) and cangrelor (AR-C69931MX), all of which demonstrated greater inhibition of ADP induced platelet aggregation compared to Clopidogrel treatment (Wiviott, 2007; Högberg et al., 2010; Nawarskas and Clark, 2011; Tan et al., 2011; Olivier et al., 2013). These antagonists have all demonstrated clear anti-thrombotic effects, both *in vivo*, with $P2Y_{12}$ blockade shown to significantly decrease P-selectin expression and platelet aggregation in response to a variety of stimuli (Ingall et al., 1999; Kauffenstein et al., 2001; Storey et al., 2002; Leon et al., 2003, 2004; Evangelista et al., 2005; Judge et al., 2010). They have also demonstrated improvements in the clinic including mortality from cardiovascular diseases, non-fatal myocardial infarction, non-fatal stroke, platelet aggregation, cAMP formation *in vitro* and other ischemic events (Perneby et al., 2007; Wiviott, 2007; Srinivasan et al., 2009; Högberg et al., 2010; Tan et al., 2011; Olivier et al., 2013). Stimulation of the $P2Y_{12}$ receptor also significantly elevates P-selectin

expression (Storey et al., 2000, 2002; Leon et al., 2003; Evangelista et al., 2005; Perneby et al., 2007),.

In addition to being coupled to AC signalling, the $P2Y_{12}$ receptor also couples to the phosphoinositol-3-kinase (PI3-K) signalling pathway through the β/γ complex. One of the main outcomes of PI3-K signalling is mobilisation of cytosolic Ca^{2+} (Hirsch et al., 2001; Kauffenstein et al., 2001; Reséndiz et al., 2003; Hardy et al., 2004; Gachet, 2006). Platelets express 4 different isoforms of PI3-K (PI3Kα, PI3Kβ, PI3Kγ and PI3Kδ) (Hirsch et al., 2001; Garcia et al., 2010), with the α and β isoforms identified as regulators of platelet aggregation (Gilio et al., 2009). In addition to the mobilisation of cytosolic Ca^{2+}, PI3-K is also involved in the activation of the integrin $\alpha_{IIb}\beta_3$, where studies have demonstrated impaired platelet aggregation through the use of specific PI3Kβ inhibitors (TGX-221) (Garcia et al., 2010). Cosemans *et al*., 2006 demonstrated that both PI3-Kβ and γ were required to perpetuate $\alpha_{IIb}\beta_3$ activation and the maintenance of platelet aggregates (Cosemans et al., 2006).

A potential role for $P2Y_{12}$ receptor involvement in inflammation has recently been demonstrated through attenuation of LTE_4 induced pulmonary inflammation in the mouse following treatment with $P2Y_{12}$ receptor antagonists (Paruchuri et al., 2009). In contrast, additional studies have shown that LPS induced systemic inflammation becomes more severe in the absence of $P2Y_{12}$ receptors (Liverani et al., 2014), with $P2Y_{12}$ receptor blockade using Clopidogrel also potentiating inflammation in a rat model of peptidoglycan-polysaccharide (PG-PS) induced arthritis (Garcia et al., 2011). This demonstrates a clear need to further investigate the role of the $P2Y_{12}$ receptor in inflammatory conditions.

1.7 Aims and Objectives

1) To investigate whether modulation of platelet purinergic signalling can influence leukocyte recruitment in a murine model of allergic pulmonary inflammation.

Leukocyte recruitment is a critical event in the development of chronic inflammation in patients with asthma. Numerous animal models have highlighted the critical role platelets play in orchestrating this response following exposure to allergen. Given the importance purinergic receptor signalling plays in the haemostatic role of platelets and the ability of the purinergic receptors to modulate P-selectin expression and the formation of platelet-leukocyte conjugates, two processes key to the induction of leukocyte recruitment following allergen challenge, a murine model of ovalbumin-induced pulmonary lung inflammation was used to evaluate the differential impact of each of the purinergic receptors on pulmonary leukocyte recruitment

Furthermore, to confirm that any effect demonstrated by purinergic receptor modulation is platelet specific, mice sensitised to OVA were specifically depleted of platelets via a non-immunological method of platelet depletion using the bone marrow cell specific toxin busulfan, inducing a gradual, long term, non-immune thrombocytopenia that can be replenished through platelet reinfusion from donor mice treated *ex vivo* with purinergic receptor antagonists

2) Identify the effects of purinergic receptor blockade on parameters of platelet activation. Extending from the objectives described above, the impact of purinergic receptor signalling blockade on parameters of platelet function including P-selectin expression, platelet-leukocyte conjugation, platelet-induced leukocyte chemotaxis, and platelet aggregation was evaluated. Previous work has highlighted an importance of both the $P2Y_1$ and $P2Y_{12}$ receptors on platelet P-selectin expression and platelet aggregation. My aim was to evaluate the differential influence of purinergic receptor activation on parameters of platelet activation and to identify potential differences between pathways associated with thrombosis and haemostasis and pathways attributed to inflammation.

3) Dissecting a possible dichotomy in platelet activation and function with relation to both haemostatic and inflammatory stimuli. Using a range of inflammatory (MDC, SDF-1α, f-MLP, 5-HT, IL-1β and LPS) and haemostatic stimuli (ADP, Adrenaline, TxA_2 and TRAP), I have investigated the impact of stimulation of different platelet receptors on classical assays of platelet function including P-selectin expression, platelet-leukocyte aggregate formation, platelet migration and platelet aggregation. This was performed in an attempt to shed further light on the mechanisms underlying the dichotomy of platelet functions described by differential platelet function as a result of stimulation by either inflammatory of haemostatic stimuli.

Chapter II

Materials and Methods

2.1 Materials.

Balb/C mice	Harlan Laboratories, Oxfordshire, UK.
Chicken Egg Ovalbumin (Grade V)	Sigma-Aldrich Ltd, Dorset, UK.
Aluminium Hydroxide	Sanofi Winthrop Ltd. Brazil.
0.9% Saline	Baxter Healthcare Ltd. UK
Urethane	Sigma-Aldrich, Dorset, UK.
DMSO	Sigma-Aldrich Ltd, Dorset, UK
Apyrase	Sigma-Aldrich Ltd, Dorset, UK
MRS2179	Tocris Bioscience, Bristol, UK
MRS2395	Sigma-Aldrich Ltd, Dorset, UK
MRS2500	Tocris Bioscience, Bristol, UK
AR-C66096	Tocris Bioscience, Bristol, UK
NF-279	Tocris Bioscience, Bristol, UK
AMD3100	Tocris Bioscience, Bristol, UK
MRS2365	Tocris Bioscience, Bristol, UK
GSK429286	Tocris Bioscience, Bristol, UK
Clopidogrel	Tocris Bioscience, Bristol, UK
C-021	Merck Chemicals, Hertfordshire, UK
PEG400	Fischer Scientific, Loughborough, UK
Busulfan	Sigma-Aldrich Ltd, Dorset, UK
Citrate-Dextrose	Sigma-Aldrich Ltd, Dorset, UK
Murine MDC	PeproTech EC, Ltd, London, UK
Murine SDF-1α	PeproTech EC, Ltd, London, UK
Human MDC	PeproTech EC, Ltd, London, UK
Human SDF-1α	PeproTech EC, Ltd, London, UK
f-MLP	Sigma-Aldrich Ltd, Dorset, UK

LPS	Sigma-Aldrich Ltd, Dorset, UK
adrenaline	Sigma-Aldrich Ltd, Dorset, UK
TRAP	Tocris Bioscience, Bristol, UK
5-HT	Sigma-Aldrich Ltd, Dorset, UK
U46619	Tocris Bioscience, Bristol, UK
IL-1β	PeproTech EC, Ltd, London, UK
Adenosine bisphosphate	Sigma-Aldrich Ltd, Dorset, UK
Adenosine Triphosphate	Sigma-Aldrich Ltd, Dorset, UK
Thrombin	Sigma-Aldrich Ltd, Dorset, UK
Fibrinogen	Sigma-Aldrich Ltd, Dorset, UK
Prostaglandin E1	Sigma-Aldrich Ltd, Dorset, UK
RB40.34 P-selectin blocking antibody #561923	BD Biosciences, Oxford, UK
rat anti-mouse CD45 FITC #130-091-609	Miltenyi Biotec, Surrey, UK
rat anti-mouse CD62P FITC #561923	BD Biosciences, Oxford, UK
rat anti-mouse CD41RPE #558040	BD Biosciences, Oxford, UK
FITC mouse anti-human CD41a #555466	BD Biosciences, Oxford, UK
PE Mouse anti-human CD42a #558819	BD Biosciences, Oxford, UK
FITC Mouse anti-human CD45 #555482	BD Biosciences, Oxford, UK
PE Mouse anti-human CD62P #555524	BD Biosciences, Oxford, UK
PE Mouse IgG κ Isotype Control #555574	BD Biosciences, Oxford, UK
FITC Mouse IgG κ Isotype Control	BD Biosciences, Oxford, UK

#555573	
ab74996 (rabbit anti-$P2Y_1$)	Abcam Plc, Cambridgeshire, UK
ab82725 (rabbit anti-$P2Y_{12}$)	Abcam Plc, Cambridgeshire, UK
Alexa Fluor 594 Goat anti-rabbit IgG A21223	Invitrogen Life Sciences, Paisley, UK
Rabbit anti-RhoA (phosphoS188)	Santacruz Biotechnology, Cambridge, USA
Rabbit IgG	Santacruz Biotechnology, Cambridge, USA
Optiylyse C	Beckman Coulter Inc, Buckinghamshire, UK
Dulbecco's Modified Eagle Medium	Invitrogen Life Sciences, Paisley, UK
RPMI 1640 Media	Invitrogen Life Sciences, Paisley, UK
ChemoTx® Transwell Migration System 101-3, 3µm Pores	NeuroProbe, Inc, USA
BD Falcon Transparent 3um Pet Insert	Becton Dickinson, Plymouth, UK
BD Falcon 24 Well companion plate	Becton Dickinson, Plymouth, UK
Flow-count Fluorospheres	Beckman Coulter Inc. Buckinghamshire, UK
Methylene Blue Stain	Sigma-Aldrich Ltd, Dorset, UK
Stromatol Stain	Mascia Brunelli, Italy
Reastain Quick-Diff Kit	Reagena, IBG Immucor Ltd, UK

2.2 Animals

Animal handling, care and experimental use were conducted in accordance with regulations described by The Animals (Scientific Procedures) Act (ASPA) 1986. All animals were housed under standard conditions of 22 ± 2°C and a 12:12 light:dark cycle. Pelleted diet and water was provided *ad libitum*. Soft bedding of wood shavings and paper material was provided in addition to plastic domes for environmental enrichment. All animals were sacrificed through administration of terminal 25% urethane anaesthesisa. Female Balb/c mice (20-25g) were procured from Charles River or Harlan UK laboratories. All animals were provided with a minimum acclimatisation period of 3 days upon arrival.

In vivo Animal studies

2.3.1 Allergen Induced Pulmonary Leukocyte Recruitment

In order to define optimal conditions for the induction of leukocyte recruitment following local allergen challenge to the lung, female Balb/C mice were exposed to 4 different sensitisation protocols.

The sensitisation protocols involved 3 intra-peritoneal doses of chicken egg ovalbumin (OVA) at either 10µg/mouse or 30µg/mouse dissolved in a solution saturated with $AlOH_2$ over a 2 week period. Subsequently, animals were exposed to nebulised OVA (3%) for 30 minutes either once or twice daily for 3 consecutive days. Formulation of OVA sensitisation doses per mouse are described below in table 2.1

	µl/mouse
5mg/ml OVA	6
Saline	384
AlOH	16

Table 3 OVA sensitisation formulation

2.3.1.1 Sensitisation Protocol for Allergen Induced Pulmonary Leukocyte Recruitment

Mice were sensitised to OVA (10µg or 30µg, 0.4ml, i.p.) on days 0, 4 and 10, with subsequent challenge to nebulised (DeVillbisS 99, HCE, UK) 3% OVA once or twice daily on days 14, 15 and 16. (Figure 2.1)

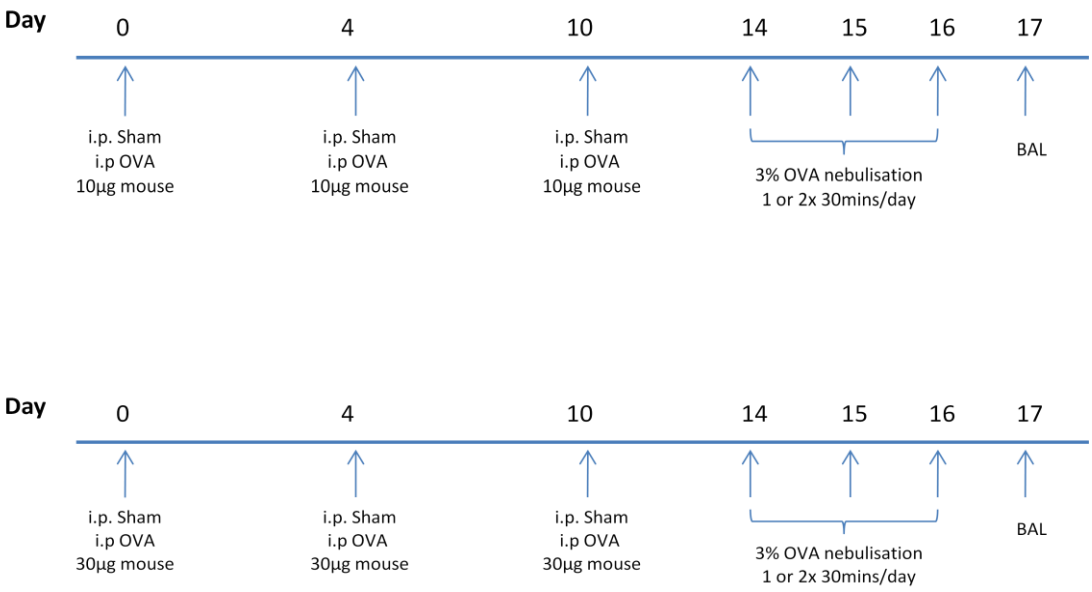


Figure 2. 1 Murine model of allergic pulmonary inflammation: A protocol for sensitisation

2.3.2 Bronchoalveolar Lavage

Twenty four hours post final challenge to OVA, mice were terminally anaesthetised with 25% urethane administered *intra-peritoneally (i.p.)* (in saline). Once anaesthetised, a small incision into the midline of the throat was made and the trachea exposed through blunt dissection, the trachea was subsequently cannulated. Once cannulated, three 0.5ml aliquots of warm saline were injected into the lung with the resulting fluid being withdrawn using a 1ml syringe and then placed on ice.

2.3.3 Total Leukocyte Counts

Total cell counts were counted from Broncho-alveolar lavage (BAL) fluid obtained. Each sample was diluted 1:1 with hemolysis solution (0.1% methyline blue in 1% acetic acid). Cell counts were then quantified using a Neubauer haemocytometer under a x20 objective.

2.3.4 Differential Leukocyte Quantification

150µl BAL fluid was placed into filters fixed onto cytospin slides and centrifuged in a Shandon Cytospin 3 at 1000rpm for 1 minute. The slides were allowed to dry and subsequently stained using a Reastain® Quick-Diff kit and coverslipped with DPX mountant. 200 cells were then counted over a representative area from each slide and the percentages of macrophages, neutrophils, lymphocytes and eosinophils determined. The total cell counts and the percentages of each differential cell type were then used to calculate the number of individual leukocytes per ml of BAL fluid.

2.3.5 Effects of ADP on Allergen Induced Pulmonary Leukocyte Recruitment in Mice

Experiments were carried out via the administration of systemic Apyrase (100u/ml, 0.1ml/mouse) though intra-venous injection, thirty minutes prior to exposure to nebulised OVA on days 14, 15 and 16. 0.1ml intra-venous saline was administered to control mice. 24 hours post final exposure to nebulised OVA (day 17), BAL fluid was taken as described in *2.3.2* and processed as described in *2.3.3-2.3.4.*

2.3.6 Effects of Purinergic Receptor Antagonists on Pulmonary Leukocyte Recruitment in Mice

Experiments investigating the impact of purinergic receptor antagonists on acute allergen induced pulmonary leukocyte recruitment were carried out through the administration of competitive $P2Y_{1,}$ $P2Y_{12}$ and $P2X_1$ receptor antagonists. The first $P2Y_1$ antagonist, the ADP analogue MRS2179 was dosed at 3, 10 and 30mg/kg i.v, (N^6-methyl-2'-deoxyadenosine 3',5'-bisphosphate). Phosphate substitutions at the 2' or 3' position results in a loss of agonist activity without impairing receptor affinity to the $P2Y_1$, resulting in a highly selective $P2Y_1$ receptor antagonist with a dissociation constant (K_D) of 109 ± 18nM and an IC_{50} value of 109 ± 22 nM towards ADP induced aggregation (Boyer et al., 1998; Baurand et al., 2001; Hechler et al., 2006). The second $P2Y_1$ receptor antagonist used was the second ADP analogue MRS2500 which was dosed at 0.1, 1, 3mg/kg i.v., (2-Iodo-N^6-methyl-*(N)*-methanocarba-2'-deoxyadenosine-3',5'-bisphosphate). Substitution of the ribose moiety with a methanocarba-ring system and with Iodo substitution of the 2' position significantly enhances affinity for the $P2Y_1$ receptor demonstrated by a K_D of 0.78nM and an IC_{50} value of 0.95nM towards ADP induced aggregation (Kim et al., 2003; Cattaneo et al., 2004).

Further experiments investigated the administration of the competitive $P2Y_{12}$ receptor antagonists MRS2395 (1, 3 and 10mg/kg, i.p) and AR-C66096 (0.1, 1 and 3mg/kg, *i.v.*). MRS2395 (2-2-Dimethyl-propionic acid 3-(2-chloro-6-methylaminopurin-9-yl)-2-(2,2-dimethyl-propionuloxymethyl-propyl ester) is an acyclic analogue of ADP with substitution of the phosphate with di(phenylacetyl) groups specifying affinity for the $P2Y_{12}$ meditated inhibition of adenylyl cyclase pathway over $P2Y_1$ mediated activation of PLC, demonstrated by a K_d of 3.6μM and a IC_{50} value of 4.6μM towards ADP induced platelet aggregation (Burnstock and Verkhratsky, 2012).

The second $P2Y_{12}$ receptor antagonist used was the ATP analogue AR-C66096 (2-(Propyltio) adenosine-5'-*O*-(β-γ-difluoromethylene) triphosphate), which provided strong and selective activity for the $P2Y_{12}$ receptor demonstrated by a K_d value of 2.5nM and a IC_{50} value of 8.16nM towards ADP induced platelet aggregation (Humphries et al., 1994; Ingall et al., 1999). Furthermore, subsitution of the anhydride link with a β-γ-methylene link in the phosphate chain prevents the conversion of the ATP analogue into ADP via activity of ectonucleotidase enzymes resulting in prolonged activity (Humphries et al., 1994; Ingall et al., 1999).

The final $P2Y_{12}$ receptor antagonist used was the thienopyridine Clopidogrel ((S)-(+)-Methyl 2-(4,5,6,7- tetrahydrothieno [3,2-*c*] pyridin-5-yl) - 2 - (2-chlorophenyl) acetate), known for its activity as a selective $P2Y_{12}$ receptor antagonist, demonstrated by a K_D value of 12μM and a IC_{50} value of 1.9 ± 0.3μM towards ADP induced platelet aggregation (Weber et al., 1999). In combination with studies using MRS2395, AR-C66096 and Clopidogrel, the effects of the $P2Y_{12}$ on parameters of platelet activation can be reliably investigated.

In addition to the $P2Y_1$ and $P2Y_{12}$ receptor antagonists, a final $P2X_1$ specific receptor antagonist NF-279 was used (0.1, 1 and 3 mg/kg *i.v.*). NF-279 (8-8'-[Carbonyl*bis*(imino-4,1-phenylenecarbonylimino-4,1-phenylenecarbonyliminio)]*bis*-1,3,5-naphthalenetrisulfonic acid) is a novel suramin-related compound which demonstrates high potency and selectivity for the $P2X_1$ receptor as demonstrated by a K_d of 19nM (Damer et al., 1998; Kügelgen and Bonn, 2008).

All drugs were administered 30 minutes before exposure to nebulised OVA on days 14, 15 and 16. 0.1ml intravenous saline was administered as a control for all experiments except for MRS2395 where a vehicle of 0.1% DMSO was administered via intra-peritoneal injection. BAL fluid was taken as described in *2.2.2* 24 hours post final allergen exposure (day 17) and processed.

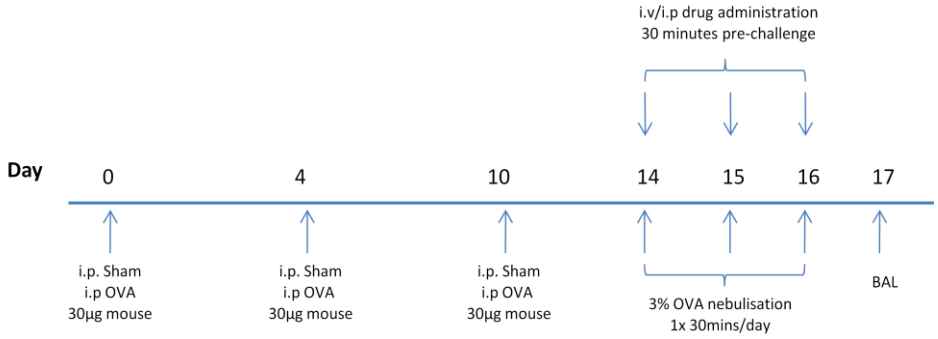


Figure 2. 2 Drug administration protocol in a murine model of pulmonary leukocyte recruitment

2.4 Effects of Platelet Depletion on a Murine Model of Allergic Inflammation

2.4.1 A Protocol of Murine Platelet Depletion

The depletion of the population of circulating platelets was induced using the bone marrow specific toxin busulfan inducing a gradual, long term state of thrombocytopenia. This mechanism of platelet depletion allows the reversal of the thrombocytopenia through platelet transfusion demonstrated in later experiments.

Busulfan was prepared in PEG400 (20mg/ml) at 60°C for 1 hour then diluted 1/8 with warm saline prior to intra-peritoneal injection (0.2ml) giving a final dose of 0.1mg/kg. The following protocol was subsequently used to investigate the impact of platelet depletion on leukocyte recruitment (Pitchford et al., 2003b, 2005).

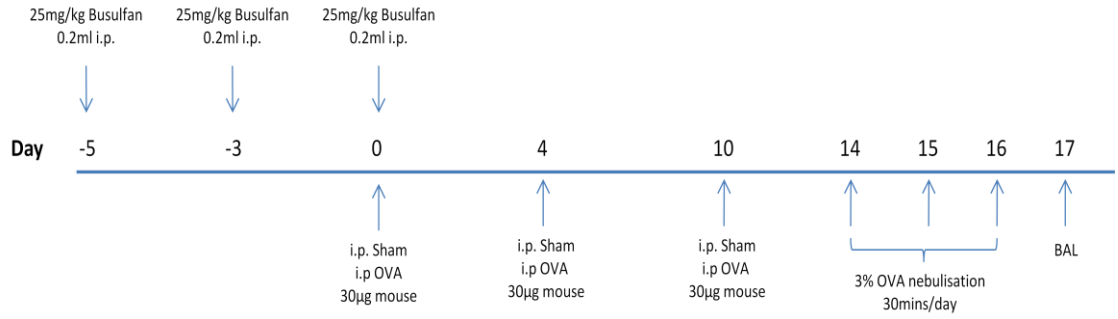


Figure 2. 3 Busulfan Induced Platelet Depletion Protocol

BAL fluid was taken 24 hours post final allergen challenge and processed as previously described. On day 14 prior to the first exposure to nebulised OVA, blood was collected through a tail vein bleed and diluted 1:100 in the haematological stain Stromatol. Circulating platelet numbers were quantified using a Neubauer haemocytometer under an x40 objective and phase contrast, on a Zeiss Axioskop upright microscope to ensure an adequate depletion of circulating platelets.

2.4.2 Platelet Reinfusion of Thrombocytopenic Mice

Platelet rich plasma (PRP) was isolated from previously sensitised mice via the collection of blood through cardiac puncture, mixing with acid-citrate dextrose (ACD) solution at a ratio of 1:9 parts blood. Blood was centrifuged at 100 g for 10 minutes followed by a second additional centrifugation step at 120 g for 5 minutes to isolate PRP. PRP was then treated with a saturating concentration of PGE_1 (10µl, 2.5mM), and subsequently centrifuged again at 900 g for 6 minutes. The resulting platelet pellet was resuspended in PBS and injected into thrombocytopenic mice intravenously at a volume of 0.1ml on days 14 and 15, 30 minutes prior to allergen exposure. No platelet transfusion was performed on the third day of allergen exposure (day 16). Resulting transfusion of platelets was an accumulated dose of $2.36x10^8$ platelets/ml.

Experiments investigating the impact of $P2Y_1$ and $P2Y_{12}$ receptor antagonism or Rho Kinase antagonism were performed through pre-incubation of PRP with either 100µM MRS2500, AR-C66096 or the Rho Kinase inhibitor GSK429286 for 20 minutes. PRP was then treated with a saturating concentration of PGE_1 (10µl, 2.5mM), and subsequently centrifuged again at 900 g for 6 minutes. The resulting platelet pellet was resuspended in PBS and injected into thrombocytopenic mice intravenously at a volume of 0.1ml on days 14 and 15, 30 minutes prior to allergen exposure.

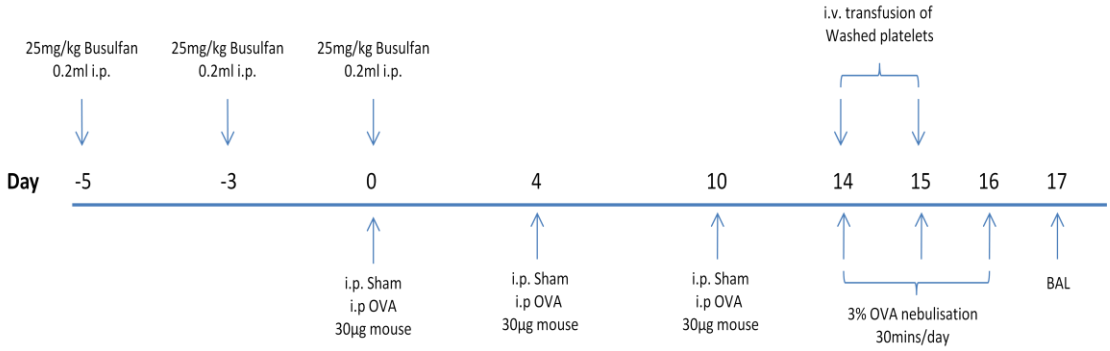


Figure 2. 4 Platelet Transfusion protocol

BAL fluid was collected 24 hours post the final exposure to nebulised OVA (day 17) and processed as previously described in *2.3.2 – 2.3.4.*

In Vitro Mouse Studies

2.5 Cell Isolation

2.5.1 Blood Collection

Female Balb/c mice were terminally anaesthetised with 0.2ml 25% w/v Urethane. Once anaesthetised, blood was collected via cardiac puncture mixing with ACD at a ratio of 1 part ACD to 9 parts Blood.

2.5.2 Washed Platelet Isolation

Citrated blood was centrifuged at 100 g for 10 minutes and the resulting PRP isolated. Blood was then centrifuged again for a further 5 minutes at 120 g and the remaining PRP collected. Saturating concentrations of PGE_1 (10µl, 2.5mM) were added to the PRP which was then centrifuged again at 900 g for 6 minutes. The resulting platelet pellet was resuspended in tyrodes buffer. Platelets were then stained in stromatol and quantified on a Neubauer haemocytometer under a x40 objective and phase contrast, on a Zeiss Axioskop upright microscope and diluted to a final concentration of $1x10^8$plts/ml in tyrodes buffer.

2.5.3.1 Sepharose Column Preparation

Sepharose columns were prepared using Sepharose C12-B (Sigma-Aldrich, Dorset, UK). Sepharose was first washed in a Buchner filter with 100% acetone. After washing with acetone, the sepharose was placed in a beaker with distilled water and mixed on a magnetic stirrer. The sepharose was then washed with distilled water through the Buchner filter. This wash cycle was repeated untill the odour of acetone

had been lost. After washing, the sepharose was stored in 10% ethanol (1 part sepharose: 2 parts ethanol) at 4°C.

When required, washed sepharose was placed into a 30ml pre-fitted reservoir column and washed with 10% ethanol until the column was half full with settled sepharose. The column is then stored in 10% ethanol at 4°C.

2.5.3.2 Gel Filtered Platelet Isolation

On the day of the experiment, the sepharose column was washed with approximately 40ml HEPEs tyrodes buffer (137mM NaCl, 2.3mM KCl, 1mM $MgCl_2.6H_2O$, 4mM $NaH_2PO_4.H_2O$, 3.1mM HEPES, 4.6mM Glucose) with 1% BSA at pH7.4.

Citrated blood was centrifuged at 100 g for 10 minutes and the resulting PRP isolated, blood was then centrifuged again for a further 5 minutes at 120 g and the remaining PRP collected. PRP was added to the top of the sepharose matrix (no less than 0.5ml, no more than 1.5ml) and small amounts of HEPES buffer added to the top of the column. Only the cloudy platelet containing media eluted from the base of the column was collected with attention paid to ensure no plasma was collected. Platelets were then stained in stromatol (1/100) and quantified on a Neubauer haemocytometer under a x40 objective and diluted to a final concentration of $1x10^8$plts/ml in HEPES tyrodes buffer.

2.5.4. Leukocyte Isolation

Following collection of blood via cardiac puncture, femurs were extracted from the mice and washed through with DMEM with supplements (1g/L glucose, 2mM glutamine, 100u/ml penicillin, 100µg/ml streptamycin, 1mM sodium pyruvate) collecting the bone marrow. Bone marrow was mixed in 15ml DMEM and

centrifuged at 300 g for 5 minutes producing a leukocyte pellet. Following centrifugation, excess DMEM was aspirated and the leukocyte pellet resuspended in 1ml DMEM. Leukocytes were diluted 1/10 in hemolysis solution (0.1% methyline blue in 1% acetic acid) and quantified in a Neubauer haemocytometer under a x20 objective. The concentration of the leukocyte suspension was then adjusted to a final working concentration of $5x10^6$cells/ml in DMEM.

2.6.1 Platelet-Induced Leukocyte Chemotaxis *in vitro*

In order to identify the correct conditions required for platelet-induced leukocyte chemotaxis, *in vitro* leukocyte chemotaxis was measured towards different chemokines including MDC and SDF-1α both in the absence and presence of platelets. The effect of OVA sensitisation was also investigated in these experiments, female balb/c mice were sensitised using a similar protocol as previously described (Figure 2.1)

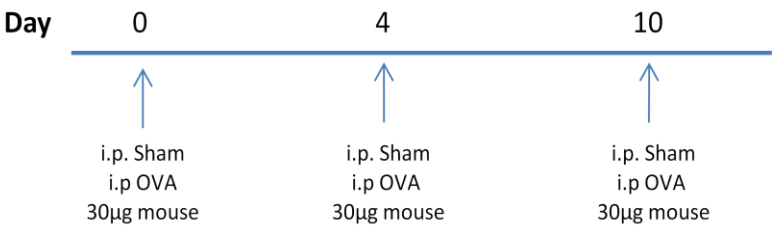


Figure 2. 5 *Ex vivo* allergen sensitisation protocol

Following a 10 day sensitisation period involving 3 doses of 30µg OVA (0.4ml, intra-peritoneal), mouse washed platelets were isolated from blood and leukocyte populations isolated from femurs as described in sections 2.5.1-2.4.4.

Platelet induced leukocyte chemotaxis was investigated using an assay based around the ChemoTx® Transwell Migration System supplied by Neuro Probe (Kornerup, et al. 2010). Chemotaxis plates with 3µm pores were blocked with 31µl tyrodes buffer + 2% foetal bovine serum (FBS) for 1 hour. After blocking, the buffer was removed and replaced with 31µl 100nM chemokine (MDC, SDF-1α, f-MLP). Platelets prepared as described previously (2.5.2) were stimulated with 100nM ADP at 37°C and washed to remove any excess ADP. Following ADP incubation, platelets were mixed at a 1:1 volume ratio (20:1 cellular ratio) with leukocytes prepared as described in 2.5.2. The chemotaxis membrane containing the 3µm pores was placed over the bottom wells containing 100nM chemokine and the 20µl platelet/leukocyte mixture applied onto the top of the membrane (Figure 2.6) and incubated for 90 minutes at 37°C.

Post 90 minute incubation, the chemotaxis membrane was removed from the top of the plate and media was collected from the bottom chamber. 10µl of the media was mixed 1:1 with hemolysis solution (0.1% methyline blue in 1% acetic acid) and leukocytes subsequently quantified in a Neubauer haemocytometer under a x20 objective. The number of leukocytes present in the bottom chamber post incubation was taken as a measure of leukocyte chemotaxis and quantified using a chemotactic index (CI).

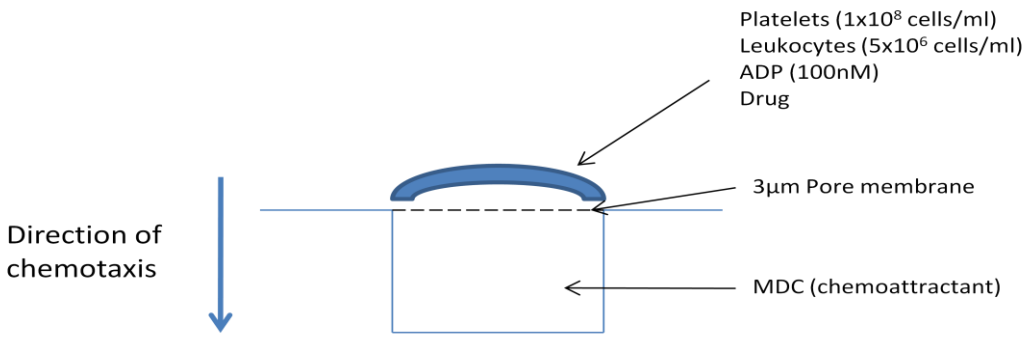


Figure 2. 6 Diagrammatic representation of platelet-induced PMN chemotaxis

2.6.2 Effects of CXCR4 and CCR4 Receptor Antagonists on Platelet-Induced Leukocyte Chemotaxis

To investigate the impact of chemokine receptor antagonists on platelet-induced leukocyte chemotaxis, platelets were pre-incubated with either the CXCR4 antagonist AMD3100 (1, 10, 100, 1000nM) or the CCR4 receptor antagonist C-021 (1, 10, 100, 1000nM) for 10 minutes at room temperature. Platelets were then centrifuged at 900 g for 6 minutes and resuspended in tyrodes buffer. Drug treated platelets were subsequently stimulated with 100nM ADP and chemotaxis measured as previously described (2.5.3).

2.6.3 Effects of Purinergic Receptor Antagonism on Platelet-Induced Leukocyte Chemotaxis

Experiments investigating the impact of purinergic receptor antagonists on platelet-induced leukocyte chemotaxis were performed by the pre-incubation of platelets with either the competitive $P2Y_1$ receptor antagonists MRS2179 and MRS2500, the competitive $P2Y_{12}$ receptor antagonists MRS2395 and AR-C66096 or the $P2X_1$

receptor antagonist NF-279. All drugs were treated at 1, 10, 100 and 1000nM for 10 minutes at room temperature with a saline vehicle used as a control. Platelets were then centrifuged at 900 g for 6 minutes and resuspended in tyrodes buffer. Drug treated platelets were subsequently stimulated with 100nM ADP and chemotaxis measured as previously described (2.5.3).

2.6.4 Effects of Rho Kinase Inhibition and P-selectin Blockade on Platelet-Induced Leukocyte Chemotaxis

Experiments investigating the impact of purinergic receptor signalling on platelet-induced leukocyte chemotaxis were performed by the pre-incubation of platelets with the competitive Rho Kinase receptor antagonist GSK429286 or RB40.34 (A P-selectin blocking antibody). Platelets were treated with 0.1, 1, 10, 100μM for 20 minutes at room temperature with a 0.01% DMSO vehicle used as a control. Platelets were then centrifuged at 900 g for 6 minutes and resuspended in tyrodes buffer. Drug treated platelets were subsequently stimulated with 100nM ADP or 1μM of the $P2Y_1$ receptor specific agonist MRS2365 and chemotaxis measured as previously described (2.4.3).

2.7 Platelet Activation Demonstrated Through P-selectin Expression

Platelets were isolated from whole blood via gel filtration as described in section 2.5.1 and 2.5.3. Gel filtered platelets were pre-treated with vehicle, 1, 10, 100, 1000nM MRS2500 or 1, 10, 100, 1000nM AR-C66096 for 15 minutes at room temperature. Following antagonist treatment, platelets were stimulated with 10μM ADP for 30 minutes at room temperature. After incubation, 10μl stimulated platelets were added to FACs tubes containing 100μl PBS and 5μl anti-CD62P-FITC and left

to incubate for a further 30 minutes at room temperature. Following incubation 500µl PBS was added to each sample and analysed on a EPICS XL-MCL s/n Y48314 flow cytometer (Beckman Coulter) by the same operator throughout the study. Data was collected on FITC fluorescence at 515nm. Live gating was used to specifically identify platelets using forward and side scatter analysing 10 000 events. Events staining for CD62P were considered to represent P-selectin expressing platelets.

Ex vivo Mouse Studies

2.8 Analysis of Platelet-Leukocyte Conjugate Formation *ex vivo*

Female Balb/c mice treated with Vehicle, 3mg/kg MRS2500 or 3mg/kg AR-C66096 were sensitised and challenged to OVA as described in figure 2.7. 6 hours post challenge, whole blood was collected via saphenous vein bleeds as described in section 2.4.1

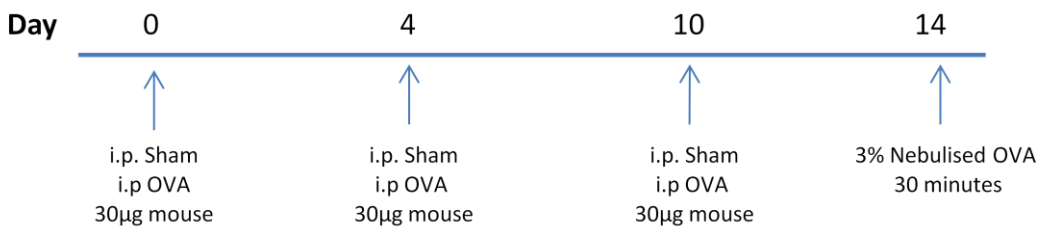


Figure 2. 7 *Ex Vivo* OVA sensitisation and challenge protocol

50µl murine whole blood were treated with saturating concentrations of the platelet specific monoclonal antibody CD41-PE and the leukocyte specific monoclonal antibody CD45-FITC were added to each sample. Blood samples were incubated with the respective antibodies for 30 minutes in the dark at 4°C. After incubation, the reaction was terminated through the addition of 500µl Optilyse C for 1 hr, used to lyse red blood cells, samples were kept for up to 1 day at 4°C before analysis. The quantification of platelet-leukocyte conjugates was then analysed on a EPICS XL-MCL s/n Y48314 flow cytometer (Beckman Coulter) by the same operator throughout the study. Data was collected on FITC fluorescence at 515nm and PE fluorescence at 580nm. Live gating was used to include events of a similar size to leukocytes using either forward or side scatter. Events staining for two separate antigens (CD41 and CD45) were considered to represent platelets attached to leukocytes through measurements of CD41 emission on CD45 positive events.

2.9 Allergen Induced Alterations in Surface Expression of Platelet $P2Y_1$ and $P2Y_{12}$ Receptors

25µl of platelets taken from sham or OVA sensitised mice were added to tubes and fixed with 2% paraformaldehyde (PFA) for 10 minutes. Excess PFA was then removed through a washing cycle (Samples were centrifuged 3 times at 1000 g for 5 minutes and resuspended in FACS buffer after each stage of centrifugation). Following the wash cycle, platelets were subsequently incubated with saturating concentrations of either rabbit $P2Y_1$ or $P2Y_{12}$ pAb in combination with saturating concentrations (PE)- labelled CD42 MoAb and a Alexa Fluor 594 - labelled rabbit anti-goat IgG secondary antibody. The antibodies were left to incubate with platelets for 30 minutes in the dark at 4°C. Excess unbound antibody was then removed by

repetition of the previously described wash cycle. Samples were analysed on a EPICS XL-MCL s/n Y48314 flow cytometer (Beckman Coulter) by the same operator throughout the study. Data was collected on PE fluorescence at 580nm and Alexa Fluor fluorescence at 615nm. Live gating was used to specifically identify CD41 expressing platelets using forward and side scatter analysing 10000 events. Events staining for two separate antigens (CD41 and $P2Y_1$/$P2Y_{12}$) were considered to represent platelets expressing either the $P2Y_1$ or $P2Y_{12}$ receptors.

2.10 Platelet Aggregation

2.10.1 *In vitro* Platelet Aggregation

Gel filtered platelets (225µl) ($2x10^8$ plts/ml) were prepared as described in section 2.5.3 and were preincubated with varying concentrations of MRS3500, AR-C66096 or clopidogrel in microcuvettes placed in holding wells for 10 minutes at 37°C. Small magnetic stir bars were added into each microcuvette and the stir speed set to 1000rpm on the aggregometer. After incubation with drugs, platelets were placed into the recording wells. Platelet aggregation was then induced through stimulation of platelets with an appropriate agonist (i.e. ADP/thrombin). The effects of specific agonists and drug treatment on platelet aggregation was measured using a Bio/Data Corporation optical aggregometer, measuring platelet aggregation using the turbidometric method developed by Born (Born., 1963).

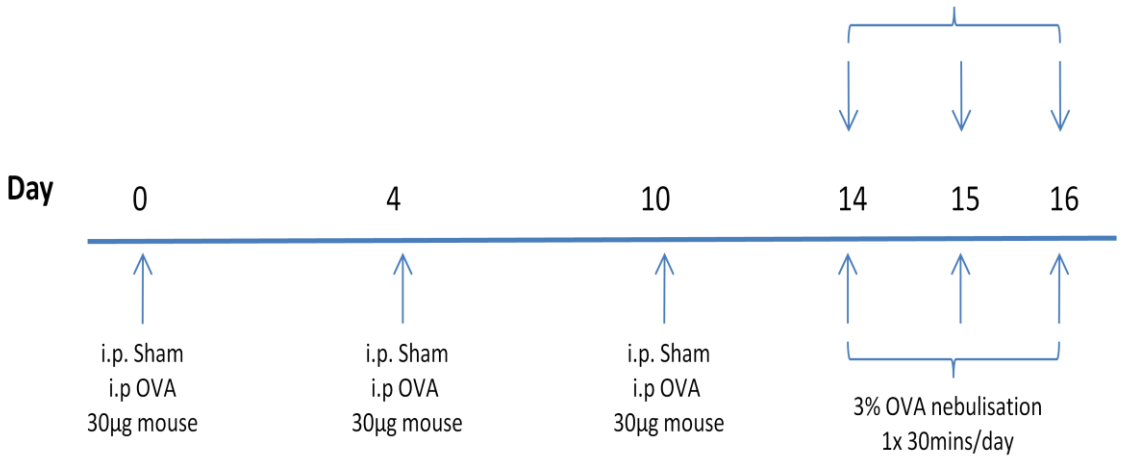
2.10.2 The Effects of Allergen Challenge on Platelet Aggregation *ex vivo*

Female Balb/c mice were sensitised and challenged to OVA as previously described (Figure 2.7). 30 minutes prior to allergen challenge mice were dosed intravenously with either 3mg/kg MRS2500 or AR-C66096. 6 hours post allergen challenge mice were anaesthetised with 0.3ml 25% urethane (in saline) and blood collected via cardiac puncture into a 1ml syringe, mixing with ACD at a 1:9 ratio with blood. Washed platelets were isolated as previously described in 2.5.3 and subsequent aggregation of washed platelets performed as described previously (2.10).

2.11 Investigating the Role of the Small GTPase RhoA on Parameters of Platelet Activation

2.11.1 *In vivo* Stimulation for RhoA Expression Quantification

Female Balb/c mice were sensitised and challenged to OVA as described below in figure 2.8. 30 minutes before allergen challenges on days 14, 15 and 16 animals were treated with either vehicle, 3mg/kg MRS2500 or 3mg/kg AR-C66096. 6 hrs after the final allergen challenge, citrated blood was collected via cardiac puncture. Washed platelets were subsequently isolated from whole blood as described in 2.5.3.



2.11.2 Preparation of Cell Extracts and Western Blot

Platelet RhoA expression was investigated in platelets by immunoblotting with a rabbit anti-pRhoA Ab. Washed platelets isolated in 2.9.1 were lysed in RIPA buffer (1% Nonidet P40, 0.5% Sodiodeossicolato, 0.1% SDS, 1mM PMDF, 1mM Na_2VO_4, pH 7.4) for 30 minutes on ice. Lysates were subsequently cleared by centrifugation at 10000g for 10 minutes at 4°C. The protein contents were then quantified through a Bradford Protein assay (Bio-Rad Laboratories). Equal amounts of protein were then separated through 12% SDS-polyacrylamide gel electrophoresis and immunoblotting followed by enhanced chemiluminescence (ECL) detection, using anti-pRhoA S188 (Abcam) diluted 1:1000 in 0.01% TBS tween and 5% BSA and the anti-rabbit HRP (Biorad) diluted 1:3000 in 0.01% TBS tween and 5% milk.

Human *in vitro* Studies

2.12 Platelet Isolation

2.12.1 Sepharose Column Preparation

Sepharose columns were prepared using Sepharose C12-B (Sigma-Aldrich, Dorset, UK). Sepharose was first washed in a Buchner filter with 100% acetone. After washing with acetone, the sepharose was placed in a beaker with distilled water and mixed on a magnetic stirrer. The sepharose was then washed with distilled water through the Buchner filter. This wash cycle was repeated untill the odour of acetone had been lost. After washing, the sepharose was stored in 10% ethanol (1 part sepharose: 2 parts ethanol) at 4°C.

When required washed sepharose was placed into a 60ml pre-fitted reservoir column and washed with 10% ethanol until the column was half full with settled sepharose. The column is then stored in 10% ethanol at 4°C.

2.12.2 Platelet Isolation via Gel filtration

On the day of the experiment, the sepharose column was washed with approximately 70ml HEPEs tyrodes buffer (137mM NaCl, 2.3mM KCl, 1mM $MgCl_2.6H_2O$, 4mM $NaH_2PO_4.H_2O$, 3.1mM HEPES, 4.6mM Glucose) with 1% BSA at pH7.4.

Human citrated venous blood (isolated from volunteers who had not taken anti-platelet drugs for 10 days prior to donation) was centrifuged at 120g for 10 minutes and resulting PRP was collected. If required a second centrifugation step at 120g for 10 minutes was performed to isolate additional PRP.

PRP was added to the top of the sepharose matrix (no less than 1ml, no more than 3ml) and small amounts of HEPES buffer added to the top of the column. Only the cloudy platelet containing media eluted from the base of the column was collected with attention paid to ensure no plasma was collected. Platelets were then stained in stromatol (1/100) and quantified on a Neubauer haemocytometer under a x20 objective and diluted to a final concentration of $1x10^8$plts/ml in HEPES tyrodes buffer.

2.12.3 Washed Platelet Isolation

Human venous blood was collected in a citrated syringe (1 ACD: 9 Blood) and centrifuged at 120g for 10 minutes at 25°C, the resulting PRP was collected and the remaining blood centrifuged for a further 10 minutes at 120g and 25°C, collecting any remaining PRP. 2.5mM PGE_1 was added to PRP and subsequently centrifuged at 1300 g for 10 minutes at 25°C. The supernatant was aspirated and the platelet pellet resuspended in 1ml tyrodes buffer. 25μM PGE_1 and 50U/ml Heparin were added and the final volume made up to 10ml with tyrodes. The platelet suspension was then centrifuged at 1300 g for 10 minutes at 25°C. After centrifugation, the supernatant was aspirated and the platelet pellet resuspended in 1ml RPMI 1640 media. Platelets were then stained in stromatol (1/100) and quantified on a Neubauer haemocytometer under a x40 objective and phase contrast on a upright Zeiss Axioskop microscope and diluted to a final concentration of $1x10^8$plts/ml in RPMI 1640 media. Platelets were left for 45-60 minutes to ensure the activity of PGE_1 had worn off before used in subsequent experiments.

2.13 Platelet Activation Demonstrated through P-selectin Expression *in vitro*

Gel filtered platelets were treated with 2mM Ca^{2+} and then stimulated with different agonists (MDC, SDF-1α, 5-HT, IL-1β, f-MLP LPS, ADP, Adrenaline, U46619 or TRAP) for 30 minutes at room temperature. After incubation, 10µl stimulated platelets were added to FACs tubes containing 100µl PBS and 5µl CD62P-FITC and left to incubate for a further 30 minutes at room temperature. Following incubation 500µl PBS was added to each sample and analysed on a EPICS XL-MCL s/n Y48314 flow cytometer (Beckman Coulter) by the same operator throughout the study. Data was collected on FITC fluorescence at 515nm. Live gating was used to specifically identify platelets using forward and side scatter analysing 10 000 events. Events staining for CD62P were considered to represent P-selectin expressing platelets.

2.14 Measurement of Platelet-Leukocyte Conjugate Formation *in vitro*

50µl of whole blood were stimulated with varying different agonists (MDC, SDF-1, 5-HT, IL-1β, f-MLP LPS, ADP, Adrenaline, U46619 or TRAP) on a rotating platform (Rotatest Shaker R100, Ludcham, Speed setting 3.5) for 30 minutes at room temperature. Following stimulation, samples were treated with saturating concentrations of the platelet specific monoclonal antibody CD41-PE and the leukocyte specific monoclonal antibody CD45-FITC were added to each sample. Blood samples were incubated with the respective antibodies for 30 minutes in the dark at 4°C. After incubation, the reaction was terminated through the addition of 500µl Optilyse C for 1 hr, also responsible for the lysing of red blood cells. Samples were kept for up to 1 day at 4°C before analysis. The quantification of platelet-leukocyte conjugates was then analysed on a EPICS XL-MCL s/n Y48314 flow

cytometer (Beckman Coulter) by the same operator throughout the study. Data was collected on FITC fluorescence at 515nm and PE fluorescence at 580nm. Live gating was used to include events of a similar size to leukocytes using either forward or side scatter. Live gating was also used to detect CD45+ also emmiting CD41+ emission, representing platelets attached to leukocytes.

2.15 Measurement of Platelet Aggregation *in vitro*

Gel filtered platelets were isolated from human blood as previously described in section 2.12.1.2. All platelet samples were treated with 2mg/ml fibrinogen prior to commencing aggregation. 225µl platelets were added to microcuvettes containing magnetic stir bars and allowed time to equibrilate to 37°C. Platelet samples were subsequently stimulated with either vehicle controls of pro-inflammatory/thrombotic agonists (MDC, SDF-1α, IL-1β, f-MLP, 5-HT, LPS, ADP, Adrenaline, U46619, TRAP) for 10 minutes. Aggregation was measured using a Bio/Data Corporation optical aggregometer, measuring platelet aggregation using the turbidometric method developed by Born (Born., 1963).

2.16 Platelet Chemotaxis

Washed platelets were isolated from human venous whole blood via centrifugation described in section 2.12.1.3. Individual wells on a BD falcon cell 24 well plate (BD Biosciences) were blocked for 1 hour using 1ml tyrodes + 1% BSA. Post incubation, the blocking buffer was removed and the wells washed with 1 ml PBS.

450µl media containing each chemotactic stimuli (MDC, SDF-1α, IL-1β, f-MLP, 5-HT, LPS, adrenaline, ADP, U46619, TRAP) was added to the bottom well and 3µm pore PET membrane inserts (BD Biosciences) added to each well. 200µl of $1x10^8$ gel filtered platelets/ml were added into each of the transwell inserts and the entire plate incubated at 37°C for 90 minutes.

Post incubation, the transwell inserts were carefully removed and discarded and 100µl of the media in the bottom chamber incubated with 5µl of the platelet specific CD42b FITC labelled antibody for 30 minutes at room temperature. After incubation with the antibody, 500µl PBS and 100µl flow-count fluorospheres (Beckman Coulter, UK) were added to each sample. Samples were subsequently analysed for platelet quantification on a flow cytometer and used as a measure of platelet migration.

2.17 Statistical Analysis

Cell counts quantified from BAL fluid taken from allergic studies (total and differential cell counts) and blood samples (circulating platelets & leukocytes, FACS analysis, platelet aggregation) were expressed as mean ± SEM, and analysed using one way ANOVA followed by a subsequent Dunnet's multiple comparison post tests between different groups. Differences were considered significant if $P<0.05$ between individual groups.

In vitro chemotaxis data is expressed as a chemotactic index (CI). Data was analysed by means of one way ANOVA, followed by a subsequent Dunnet's multiple comparison test between groups. Significance differences were accepted if $P<0.05$ between individual groups compared to vehicle control groups.

Chapter III

Results I

Model Development

3.1. An Investigation into Leukocyte Recruitment in Allergen Challenged Mice

Validation studies were performed to establish a suitable sensitisation and challenge protocol for the induction of pulmonary leukocyte recruitment in mice. The variables studied were the sensitising concentration of OVA (either 0.5mg/kg or 1.5mg/kg) as well as the number exposures towards nebulised OVA (once or twice daily).

Murine sensitisation via intra-peritoneal injections of an OVA concentration of 0.5mg/kg induced significant total leukocyte recruitment to the lungs when exposed to either one or two periods of aerosolised OVA challenge daily compared to sham sensitised control mice (Sham: $0.99 \pm 0.15x10^5$ cells/ml vs. 0.5mg/kg OVA once daily: $6.10 \pm 0.83x10^5$ cells/ml, 0.5mg/kg OVA twice daily: $5.59 \pm 1.32x10^5$ cells/ml, **fig 3.1A**), a process that was replicated by elevations in individual inflammatory cell types including macrophages (Sham: $1.17 \pm 0.10x10^5$ cells/ml vs. 0.5mg/kg OVA once daily: $1.92 \pm 0.44x10^5$ cells/ml; 0.5mg/kg OVA twice daily: $2.28 \pm 1.38x10^5$ cells/ml, **fig 3.1B**), neutrophils (Sham: 0.00 ± 0.00 cells/ml vs. 0.5mg/kg OVA once daily: $0.02 \pm 0.01x10^5$ cells/ml; 0.5mg/kg OVA twice daily: $0.01 \pm 0.01x10^5$ cells/ml **fig 3.1C**), lymphocytes (Sham: 0.00 ± 0.00 cells/ml vs. 0.5mg/kg OVA once daily: $0.19 \pm 0.06x10^5$ cells/ml, $P < 0.01$; 0.5mg/kg OVA twice daily: $0.01 \pm 0.01x10^5$ cells/ml **fig 3.1D**) and eosinophils (Sham: 0.00 ± 0.00 cells/ml vs. 0.5mg/kg OVA once daily: $3.82 \pm 0.25x10^5$ cells/ml; 0.5mg/kg OVA twice daily: $3.34 \pm 0.79x10^5$ cells/ml, $P < 0.05$ **fig 3.1E**).

In addition to sensitisation with 0.5mg/kg OVA groups of mice were also sensitised with 1.5mg/kg OVA, which induced significant total leukocyte recruitment to the lungs when exposed to either once or twice daily aerosolised challenge to 3% OVA

when compared to sham sensitised controls (Sham: 0.99 ± 0.15x10^5 cells/ml vs. 1.5mg/kg OVA once daily: 7.41 ± 2.19x10^5 cells/ml, ; 1.5mg/kg OVA twice daily: 9.86 ± 1.10x10^5 cells/ml, $P < 0.01$, **fig 3.1A**). This elevation in total leukocyte infiltration was replicated in the individual inflammatory cell types of macrophages (Sham: 1.17 ± 0.10x10^5 cells/ml vs. 1.5mg/kg OVA once daily: 2.42 ± 0.47x10^5 cells/ml; 1.5mg/kg OVA twice daily: 2.16 ± 0.39x10^5 cells/ml, **fig 3.1B**), neutrophils (Sham: 0.00 ± 0.00 cells/ml vs. 1.5mg/kg OVA once daily: 0.04 ± 0.03x10^5 cells/ml; 1.5mg/kg OVA twice daily: 0.00 ± 0.00x10^5 cells/ml, **fig 3.1C**), lymphocytes (Sham: 0.00 ± 0.00 cells/ml vs. 1.5mg/kg OVA once daily: 0.03 ± 0.03x10^5 cells/ml; 1.5mg/kg OVA twice daily: 0.03 ± 0.03x10^5 cells/ml, **fig 3.1D**) and eosinophils (Sham: 0.00 ± 0.00 cells/ml vs. 1.5mg/kg OVA once daily: 4.89 ± 1.77x10^5 cells/ml; 1.5mg/kg OVA twice daily: 8.86 ±0.67, $P < 0.001$, **fig 3.1E**)

Furthermore, no significant difference in leukocyte recruitment was observed between once or twice daily provocation with 3% aerosolised OVA in any of measured cell types (total cells, macrophages, neutrophils, lymphocytes or eosinophils).

The data obtained from these experiments indicated that twice daily exposures to nebulised 3% OVA did not significantly increase leukocyte recruitment over once daily exposures, whilst sensitisation to a higher 1.5mg/kg dose of OVA produced a significantly larger leukocyte recruitment profile when compared with a 0.5mg/kg OVA sensitisation dose. Thus, further experiments recording the effects of various agents on the development of pulmonary leukocyte recruitment were performed 24 hours post final challenge using a sensitisation and challenge protocol consisting of 3 sensitisation doses of 1.5mg/kg on days 0, 4 and 10 followed by a single exposure towards 3% nebulised OVA for 30 minutes on days 14, 15 and 16.

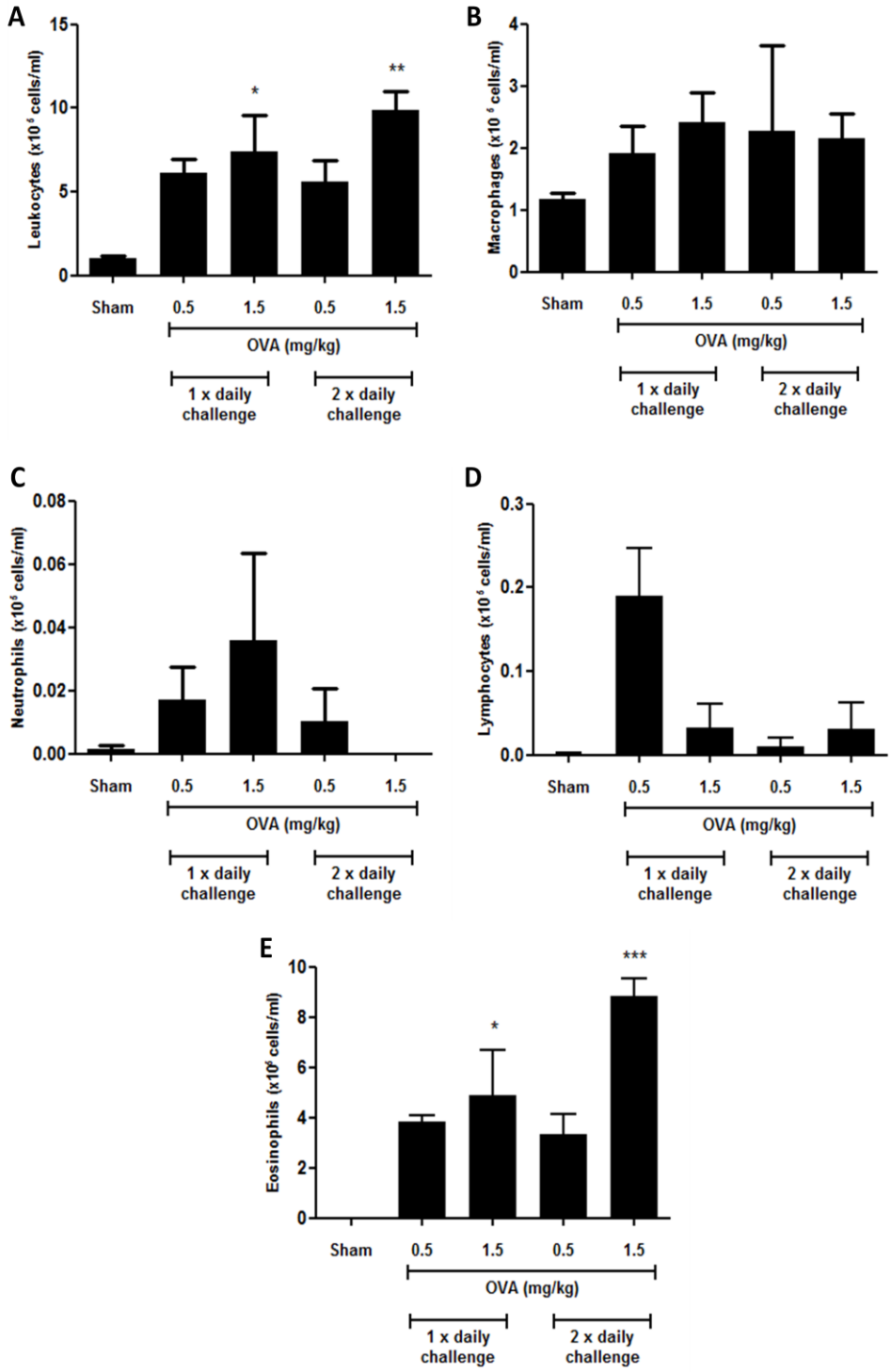


Figure 3. 1 Pulmonary Inflammatory cell recruitment using a sensitisation protocol in OVA

Mice were immunised with either 0.5/1.5mg/kg OVA (i.p.) on days 0, 4 and 10 and then exposed to nebulised OVA (3% in saline) either once or twice daily on days 14, 15 and 16. Lavage fluid was collected on day 17, 24 hours post final OVA exposure. **A** Total Leukocyte counts **B** Eosinophils. Data: Mean ± SEM. N = 4 animals per group. * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$ compared to sham controls

3.2 An Investigation into the Effects of Allergen Challenge of Circulating Platelet Dynamics over 24 hours

A number of groups have previously demonstrated that platelets are a crucial component of the machinery involved in pulmonary leukocyte recruitment following provocation in a variety of animal models including the murine model of allergic inflammation (Pitchford *et al.* 2003, 2005), in an allergic rabbit model (Coyle *et al.* 1990) and also in guinea pigs (Lellouch-Tubiana et al., 1988) with a state of mild systemic thrombocytopenia observed in asthmatic patients (Sullivan et al., 2000; Kowal et al., 2006). Given this additional parameter of platelets the impact of allergen challenge in this model of pulmonary leukocyte challenge on circulating platelet numbers were analysed from blood isolated from the tail veins at 1, 3, 6 and 24 hours post allergen challenge and compared to circulating platelet numbers prior to allergen provocation.

Circulating platelet numbers in sham-sensitised animals were unchanged throughout the time course and remained comparable to platelet numbers prior to allergen challenge. (Pre: $1.68 \pm 0.06 \times 10^9$ plts/ml; 1hr: $1.63 \pm 0.03 \times 10^9$plts/ml; 3hrs: $1.53 \pm 0.08 \times 10^9$plts/ml; 6hrs: $1.37 \pm 0.08 \times 10^9$plts/ml; 24hrs: $1.51 \pm 0.08 \times 10^9$plts/ml) (**Figure 3.2**). In contrast a mild systemic thrombocytopenia was observed in allergen challenged OVA sensitised mice over the 24 hour time course with a significant decrease in circulating platelet numbers observed at all measured time points (Pre: $1.69 \pm 0.07 \times 10^9$plts/ml; 1hr: $1.45 \pm 0.04 \times 10^9$plts/ml, $P<0.01$; 3hrs: $1.18 \pm 0.08 \times 10^9$plts/ml, $P<0.01$; 6hrs: $1.03 \pm 0.03 \times 10^9$plts/ml, $P<0.01$; 24hrs: $1.19 \pm 0.06 \times 10^9$plts/ml, $P<0.05$) (**Figure 3.2**). By 24 hours post challenge it appeared as though the circulating platelet numbers had begun to return to levels seen pre-exposure to nebulised OVA.

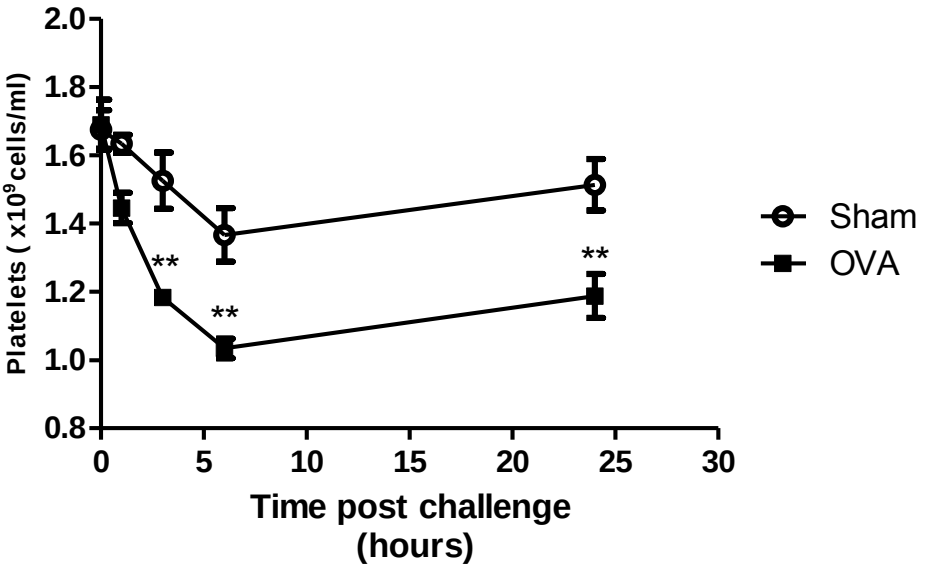


Figure 3. 2. The effects of allergen challenge on circulating platelet dynamics over 24 hours

Mice were immunised with 1.5mg/kg OVA or vehicle (i.p.) on days 0, 4 and 10 and then exposed to nebulised OVA (3% in saline) or sham controls once daily on days 14, 15 and 16. Circulating blood was collected via tail bleeds from both OVA and sham challenged groups and circulating platelets assessed pre challenge and at 1, 3, 6 and 24 hours post allergen challenge. Data: Mean ± SEM, N = 4-5 animals per group. ** = $P < 0.01$ compared to sham controls.

3.3 Validating an *in vitro* Model of Platelet P-selectin Expression

Experiments were performed to identify the most suitable protocol for the quantification of platelet P-selectin expression post stimulation following platelet isolation from whole blood.

3.3.1 ADP Induced P-selectin Expression in Washed Platelet Preparations

Washed platelet preparations isolated from whole blood via centrifugation demonstrated significant elevations in P-selectin expression following stimulation

with increasing concentrations of the platelet agonist ADP when compared to sham controls (Vehicle: 19.84 ± 0.87% vs. 0.1μM ADP: 21.45 ± 1.57%; 1μM ADP: 24.22 ± 4.42%; 10μM ADP: 36.95 ± 3.95%, $P < 0.05$; 100μM ADP: 42.23 ± 3.91%, $P < 0.05$, **fig 3.3A**). This was also reflected by ADP induced increases in platelet MFI on platelet P-selectin expression compared to vehicle controls (Sham: 0.94 ± 0.02 vs. 0.1μM ADP: 0.91 ± 0.02; 1μM ADP: 1.50 ± 0.19; 10μM ADP: 2.00 ± 0.17, $P < 0.01$; 100μM ADP: 2.17 ± 0.24, $P < 0.01$, **fig 3.3B**). It is important to note that P-selectin is not constitutively expressed on the surface of platelets and is only expressed upon platelet activation. As a result, the relatively high basal levels of platelet P-selectin expression suggests that the platelets may have become activated during the centrifugation steps required for platelet isolation from the plasma. Therefore subsequent experiments measuring P-selectin following ADP stimulation on platelets isolated through gel filtration, (a platelet isolation protocol with minimal centrifugation steps capable of inducing high levels of basal platelet P-selectin expression) were conducted.

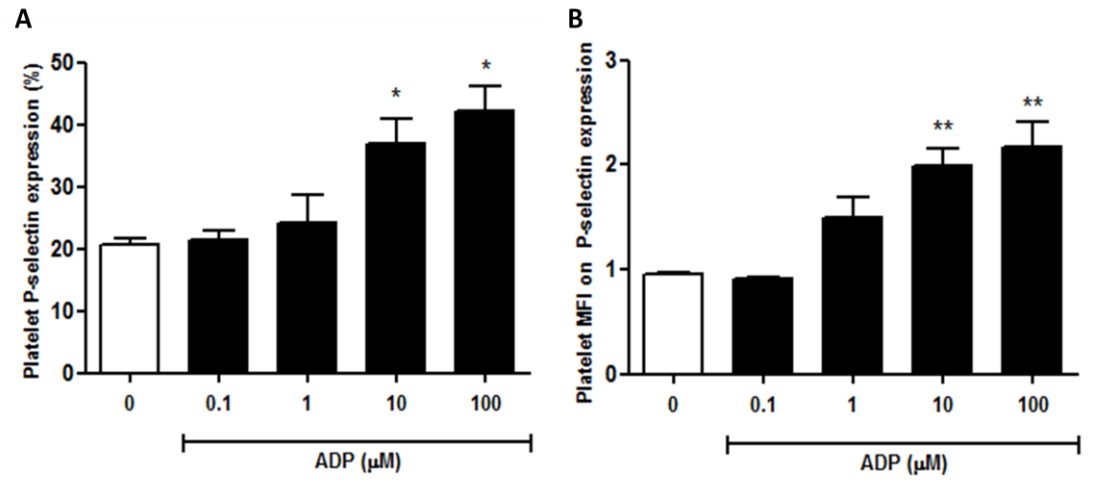


Figure 3. 3 ADP Induced P-selectin expression in Gel Filtered Platelet Preparations

Washed platelets were isolated from human blood via centrifugation and stimulated with 0.1, 1, 10 and 100μM ADP. Platelet P-selectin expression was measured via flow cytometry using a CD62P FITC labelled antibody as either the percent of platelets expressing P-selectin (**A**) or as the platelet MFI on P-selectin expression (**B**). Data expressed as Mean ± SEM. N = 3, * = $P < 0.05$, ** = $P < 0.01$ indicates significance compared to vehicle controls.

3.3.2 ADP Induced P-selectin Expression in Gel Filtered Platelet Preparations

Using platelets isolated from whole blood via gel filtration, platelet P-selectin expression was measured following stimulation with increasing concentrations of ADP. ADP induced a significant increase in surface P-selectin expression (Vehicle: 2.05 ± 0.61% vs. 0.1μM ADP: 12.08 ± 8.10%; 1μM ADP: 21.33 ± 2.15%; 10μM ADP: 32.86 ± 3.44%, $P < 0.01$; 100μM ADP: 41.46 ± 2.99%, $P < 0.01$, **figure 3.4A**) as well as inducing increases in MFI on platelet P-selectin expression (Vehicle: 0.43 ± 0.05 vs. 0.1μM ADP: 1.20 ± 0.61; 1μM ADP: 1.77 ± 0.53; 10μM ADP: 2.54 ± 0.42; 100μM ADP: 3.15 ± 0.40, $P < 0.05$, **figure 3.4B**) compared to sham controls.

Importantly, basal levels of P-selectin expression were significantly lower in platelets isolated via gel filtration when compared to platelets isolated via centrifugation (WP: 19.84 ± 0.87% vs. GFP: 2.05 ± 0.61%, $P < 0.001$), this observation was replicated in platelet MFI on P-selectin expression (WP: 0.94 ± 0.02 vs. GFP: 0.43 ± 0.05, $P < 0.05$). This indicates that there is significantly lower basal platelet activation in platelets isolated via gel filtration, therefore in future experiments measuring P-selectin expression, all platelets were isolated via gel filtration.

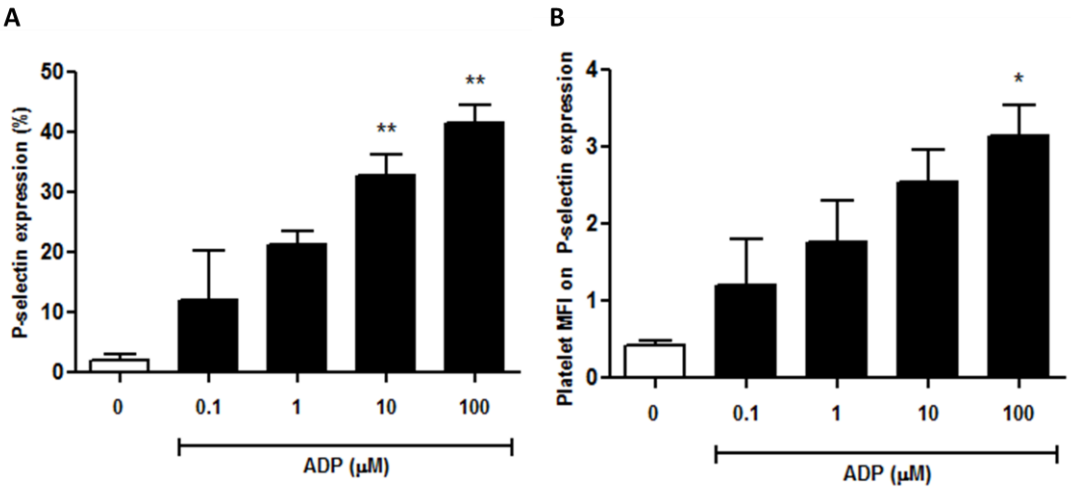


Figure 3. 4 ADP induced P-selectin expression in gel-filtered platelet preparations

Gel-filtered platelets were isolated from human blood via separation in a sepharose column and stimulated with 0.1, 1, 10 and 100μM ADP. Platelet P-selectin expression was measured via flow cytometry using a CD62P FITC labelled antibody as either the percent of platelets expressing P-selectin (**A**) or as the platelet MFI on P-selectin expression (**B**). Data expressed as Mean ± SEM. N = 3, * = $P < 0.05$, ** = $P < 0.01$ indicating significance compared to vehicle controls.

3.4 Validating an *in vitro* Model of Platelet-Induced Leukocyte Chemotaxis

Chemotaxis assays were performed on platelets and PMN cells (90% neutrophils) isolated from cardiac blood and bone marrow respectively, of allergen sensitised mice. To validate this *in vitro* model of platelet induced PMN migration, chemokines known to activate platelets were used, SDF-1α and MDC.

3.4.1 Investigating the Effects of CCR4 Receptor Antagonism on Platelet Induced PMN Chemotaxis towards MDC and SDF-1

Chemotaxis assays were performed on platelets and PMNs isolated from blood and bone marrow of allergen sensitised mice. Platelets were incubated with the CCR4 receptor antagonist C-021 prior to stimulation with 100nM ADP to confirm that migration was the result of platelet CCR4 receptor activation. Post ADP stimulation, platelets were added to PMN cells to induce their migration towards a source of MDC. No significant migration was observed with leukocytes alone (0.65 ± 0.07), leukocyte migration towards 100nM MDC (1.31 ± 0.24) or leukocytes in the presence of platelets (1.00 ± 0.15), however in the platelets and leukocytes at a 1:1 volumetric ratio (20:1 cellular ratio), platelet induced leukocyte migration was significantly enhanced (2.49 ± 0.12, $P < 0.001$, **figure 3.5A**).

Pre-incubation of platelets with 1, 10, 100 and 1000nM of C-021 resulted suppressed PMN migration towards MDC in a concentration dependent manner (Veh:2.49 ± 0.12 vs. 1nM: 2.24 ± 0.10, 10nM: 2.22 ± 0.12, 100nM: 2.04 ± 0.25, 1000nM: 1.24 ± 0.13 $P<0.001$, **figure 3.5A**) confirming that co-stimulation of platelets with MDC and ADP triggered PMN migration across the chemotactic membrane.

In contrast however the CCR4 antagonist C-021 failed to inhibit platelet-induced leukocyte migration towards SDF-1α (Veh: 1.96 ± 0.63; 1nM C-021: 2.15 ± 0.67;

10nM C-021: 2.13 ±0.54; 100nM: 2.96 ± 0.23; 1000nM C-021: 2.42 ± 0.12, **figure 3.5B**).

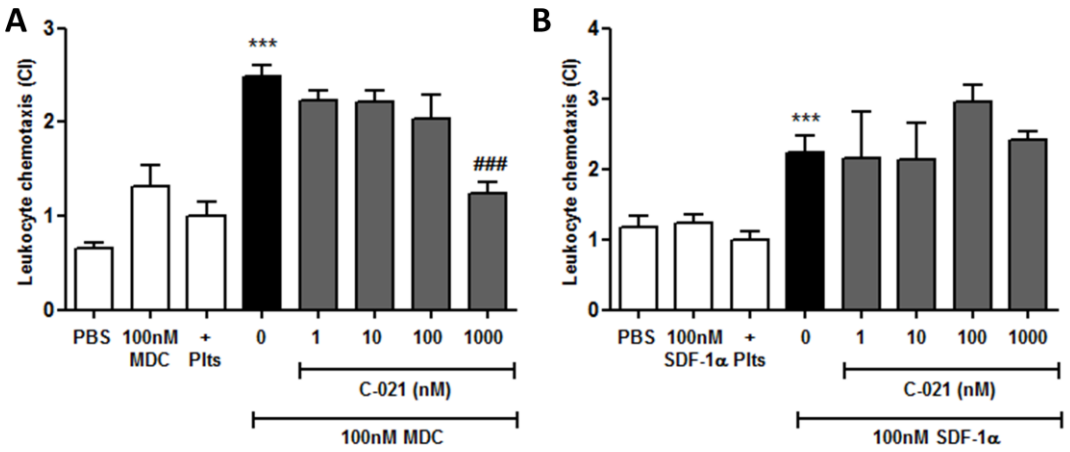


Figure 3. 5 The Effects of the CCR4 Receptor Antagonist C-021 on Platelet Induced PMN chemotaxis *in vitro*

Platelets and PMNs were isolated from citrated blood and bone marrow from female Balb/c mice. Platelets were treated with 1, 10, 100 and 1000nM C-021 and stimulated with 100nM ADP before mixing with PMN cells. The cell mixture was added to the top well of a chemotaxis chamber. PMN chemotaxis towards either 100nM MDC (A) or 100nM SDF-1α (**B**) was then quantified after a 90 minute incubation and analysed as a chemotactic index. Data are expressed as mean ± SEM. *** = $P < 0.001$ where indicated to column 3, ### = $P < 0.001$ where indicated to column 4, n =4.

3.4.2 Investigating the Effects of CXCR4 Receptor Antagonism on Platelet Induced PMN Chemotaxis Towards SDF-1α and MDC

Platelets were incubated with the CXCR4 receptor antagonist AMD3100 prior to stimulation with 100nM ADP. Post ADP stimulation, platelets were added to PMN cells to induce their migration towards a source of SDF-1α. No significant migration was observed with leukocytes alone (0.93 ± 0.12), leukocyte migration towards 100nM SDF-1α (1.04 ± 0.09) or leukocytes in the presence of platelets (1.00 ± 0.05), however in the platelets and leukocytes at a 1:1 volumetric ratio (20:1 cellular ratio),

platelet induced leukocyte migration was significantly enhanced (2.38 ± 0.09, P < 0.001, **figure 3.6A**). Pre-incubation of platelets with 1, 10, 100 and 1000nM of AMD3100 resulted suppressed PMN migration towards SDF-1α in a dose dependent manner (Veh: 2.38 ± 0.17 vs. 1nM: 2.06 ± 0.09, 10nM: 1.62 ± 0.09 P < 0.001, 100nM: 1.42 ± 0.11 P < 0.001, 1000nM: 1.03 ± 0.13 P<0.001, **figure 3.6A**) confirming that co-stimulation of platelets with SDF-1α and ADP triggered PMN migration across the chemotactic membrane. In contrast however AMD3100 failed to inhibit platelet-induced leukocyte migration towards MDC (Veh: 2.38 ± 0.17; 1nM: 2.72 ± 0.18; 10nM: 2.38 ±0.15; 100nM: 2.28 ± 0.24; 1000nM: 2.02 ± 0.16, **figure 3.6B**).

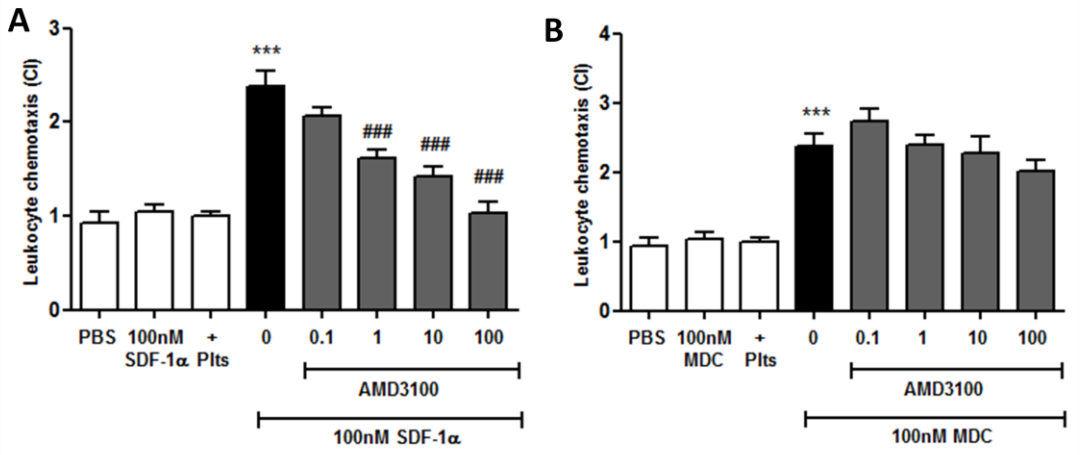


Figure 3. 6 The Effects of the CXCR4 Receptor Antagonist AMD3100 on Platelet Induced PMN Chemotaxis *in vitro*

Platelets and PMNs were isolated from citrated blood and bone marrow from female Balb/c mice. Platelets were treated with 1, 10, 100 and 1000nM C-021 and stimulated with 100nM ADP before mixing with PMN cells. The cell mixture was added to the top well of a chemotaxis chamber. PMN chemotaxis towards either 100nM SDF-1α (**A**) or 100nM MDC (**B**) was then quantified after a 90 minute incubation and analysed as a chemotactic index. Data are expressed as mean ± SEM. *** = P < 0.001 where indicated to column 3, ### = P < 0.001 where indicated to column 4, n =4.

Chapter IV

Results II

Purine Involvement in Pulmonary Leukocyte Recruitment in a Murine Model of Allergic Inflammation

4.1 An Investigation into the Involvement of ADP in Pulmonary Leukocyte Recruitment

Platelets have previously been reported as a critical cellular component in pulmonary leukocyte recruitment after allergen challenge in animal models of allergic inflammation (Lellouch-Tubiana et al., 1988; Coyle et al., 1990; Pitchford et al., 2003a). Furthermore, purinergic receptor stimulation has been reported to induce platelet activation, resulting in granular release of pro-inflammatory cytokines such as RANTES and PF-4 and upregulation of surface P-selectin expression and the formation of platelet-leukocyte conjugates (Leon et al., 2003, 2004; Straub et al., 2011), in addition to the well characterised process of aggregation (Hechler et al., 1998, 2006; Fabre et al., 1999; Léon et al., 1999; Baurand et al., 2001; Kauffenstein et al., 2001; Oury et al., 2001; Nylander et al., 2003, 2004). The elevation in P-selectin expression and the formation of platelet-leukocyte conjugates suggests a potential role of platelet purinergic stimulation in the platelet P-selectin dependent process of pulmonary leukocyte recruitment (Pitchford et al., 2003a, 2005). Therefore experiments were performed investigating the impact of purinergic receptor stimulation in this murine model of allergic inflammation.

The enzyme Apyrase which is responsible for the degradation of active ATP and ADP into inactive AMP was used in order to evaluate the impact of the purines on pulmonary leukocyte recruitment using this murine model of allergic pulmonary inflammation validated in section 3.1. Apyrase (100u/ml), dosed daily, intravenously 30 minutes before each exposure to nebulised OVA significantly suppressed allergen induced total leukocyte infiltration into the BAL (Sham: $20.00 \pm 2.08 \times 10^4$ cells/ml; OVA: $61.33 \pm 18.30 \times 10^4$ cells/ml vs. 100u/ml Apyrase: $27.00 \pm 2.02 \times 10^4$ cells/ml, $P < 0.001$) (**Figure 4.1A**).

Allergen induced elevations in total leukocyte infiltration and subsequent attenuation of infiltration following apyrase treatment was replicated in the differential cell count numbers of macrophages (Sham: 19.87 ± 2.08x10^4 cells/ml; OVA:29.67 ± 1.87x10^4 cells/ml, $P < 0.001$ vs. Apyrase: 20.55 ± 1.41, $P < 0.001$), neutrophils (Sham: 0.09 ± 0.05x10^4 cells/ml; OVA: 1.80 ± 0.59x10^4 cells/ml, $P < 0.001$ vs. Apyrase: 1.29 ± 0.74, $P < 0.001$), lymphocytes (Sham: 0.04 ± 0.04x10^4 cells/ml; OVA: 1.07 ± 0.29x10^4 cells/ml, $P < 0.001$ vs. Apyrase: 0.04 ± 0.04, $P < 0.001$) and eosinophils (Sham: 0.00 ± 0.00 cells/ml; OVA: 34.29 ± 4.02x10^4 cells/ml, $P < 0.001$ vs. Apyrase: 5.12 ± 1.07, $P < 0.001$) (**Figure 4.1B)**

This attenuation of total and differential cell infiltration into the BAL fluid following nebulised provocation to OVA strongly implicates both ATP and ADP as necessary in pulmonary leukocyte recruitment. Given that previous studies have reported that pulmonary leukocyte recruitment in this model is also platelet dependent the impact of the individual platelet purine receptors ($P2Y_1$, $P2Y_{12}$ and $P2X_1$) were subsequently investigated.

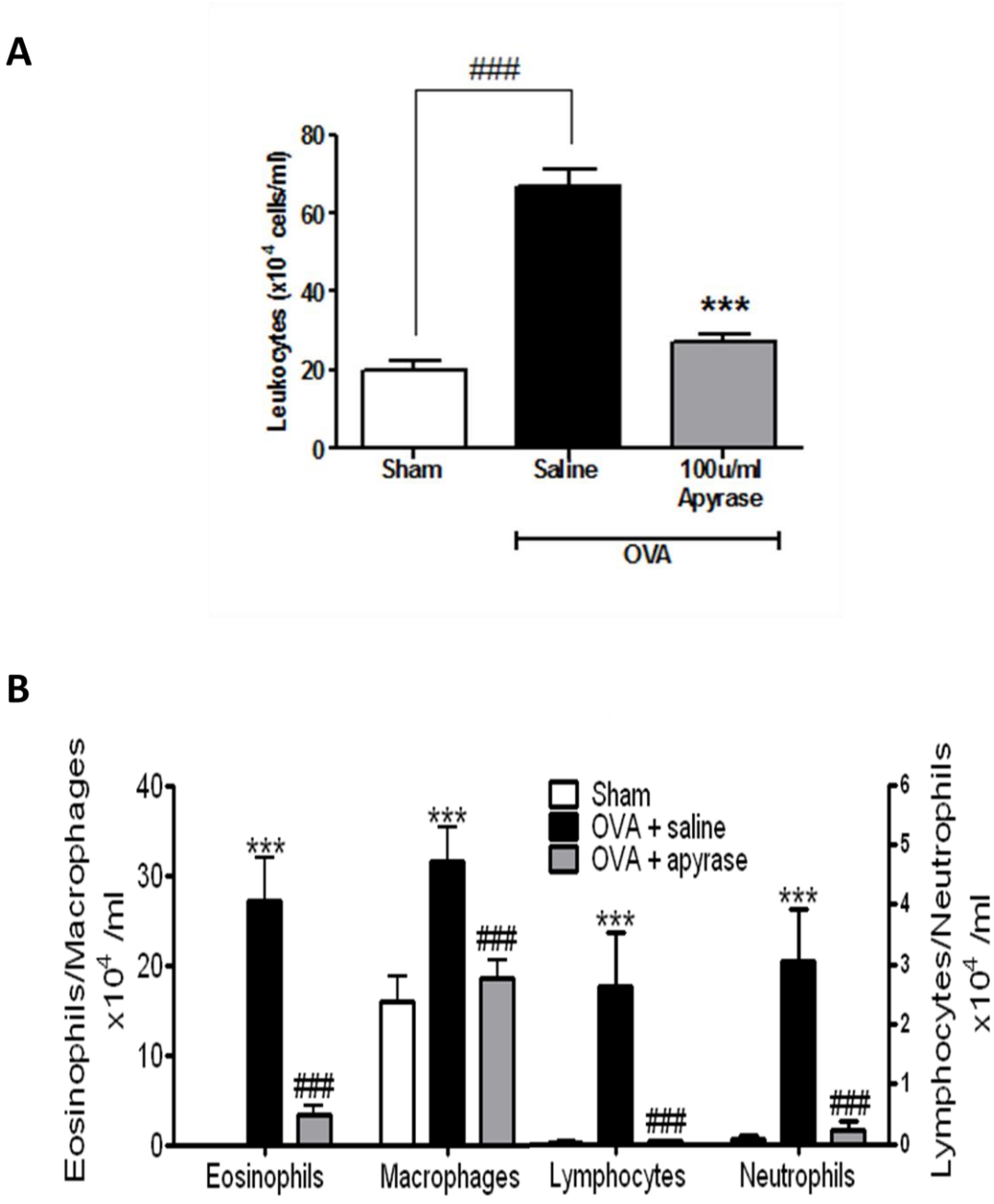


Figure 4. 1 The Effects of Apyrase of Pulmonary Leukocyte Recruitment

Mice were subjected to a two week sensitisation (1.5mg/kg OVA) and allergen exposure protocol. Apyrase was administered prior to allergen exposure on days 14, 15 and 16 (100u/ml intravenously). Lavage fluid was collected 24 hours post final allergen Exposure (day 17). **A** Total BAL Leukocytes **B** Differential Cell counts. Data: Mean ± SEM, N= 4 animals per group. * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$ compared to sham control, # = $P < 0.05$, ## = < 0.01, ### = $P < 0.001$ compared to OVA challenged mice.

4.2 An Investigation into the Effects of Purinergic Receptor Antagonism on Pulmonary Leukocyte Recruitment in a Murine Model of Allergic Inflammation

In 4.1, experiments involving the treatment of mice with Apyrase demonstrated significant attenuation of pulmonary leukocyte recruitment in a murine model of allergic inflammation thus implicating the purines in the initiation of pulmonary leukocyte recruitment. Therefore, the roles of antagonists specific for each of the purinergic receptors stimulated by adenosine phosphates on the platelet surface, including the $P2Y_1$ (MRS2179, MRS2500) and $P2Y_{12}$ (MRS2395, AR-C66096) receptors whose endogenous agonist is ADP and the ATP sensitive $P2X_1$ receptor (NF-279) were investigated, given that leukocyte recruitment is platelet dependent in this model (Lellouch-Tubiana et al., 1988; Coyle et al., 1990; Pitchford et al., 2003b, 2005)

4.2.1.An Investigation into the Effects of the $P2Y_1$ Receptor Antagonist MRS2179 on Pulmonary Leukocyte Recruitment

Female Balb/c mice were sensitised to OVA using a sensitisation protocol involving 3 intra-peritoneal injections of 30µg/mouse OVA or sham sensitised controls on days 0, 4 and 10. OVA sensitised mice were then pre-treated with vehicle or 3, 10 and 30mg/kg MRS2179 *i.p.* (an ADP analogue demonstrating selective and competitive antagonism for the $P2Y_1$ receptor) 30 minutes prior to allergen challenge on days 14, 15 and 16. Twenty-four hours post final challenge (Day 17) all animals were culled and BAL fluid collected.

Sensitisation and subsequent challenge to OVA resulted in significant elevation in total leukocyte recruitment into BAL fluid (Sham: $1.33 \pm 0.01 \times 10^5$ cells/ml vs.

OVA: 3.58 ± 0.25x10^5 cells/ml, P < 0.001) which was significantly attenuated by daily intravenous administration of MRS2179 in a dose-dependent manner when treated before exposure to nebulised OVA, as demonstrated by the top concentration of 30mg/kg MRS2179 (Vehicle: 3.58 ± 0.25x10^5 cells/ml vs. 30mg/kg MRS2179: 1.73 ± 0.11x10^5 cells/ml, P < 0.001) (**Figure 4.2A**). At 30mg/kg of MRS2179, differential leukocyte analysis of cell infiltrate revealed a significant reduction in macrophages counts (13.25 ± 1.04x10^4 cells/ml vs. OVA: 22.06 ± 4.80x10^4 cells/ml, P < 0.001; 30mg/kg MRS2179: 15.38 ± 0.84x10^4 cells/ml P < 0.001), neutrophil counts (Sham: 0.08 ± 0.03x10^4 cells/ml vs OVA: 1.21 ± 0.21x10^4 cells/ml P < 0.001; 30mg/kg MRS2179: 0.57 ± 0.17x10^4 cells/ml, P < 0.05), lymphocyte counts (Sham: 0.00 ± 0.00 cells/ml vs. OVA: 0.48 ± 0.08x10^4 cells/ml, P < 0.001; 30mg/kg MRS2179: 0.04 ± 0.03x10^4 cells/ml, P < 0.001) and eosinophil counts (Sham: 0.00 ± 0.00 vs. OVA: 13.54 ± 4.12x10^4 cells/ml, P < 0.001; 30mg/kg MRS2179: 3.29 ± 0.94x10^4 cells/ml, P < 0.001) (**Figure 4.2B**).

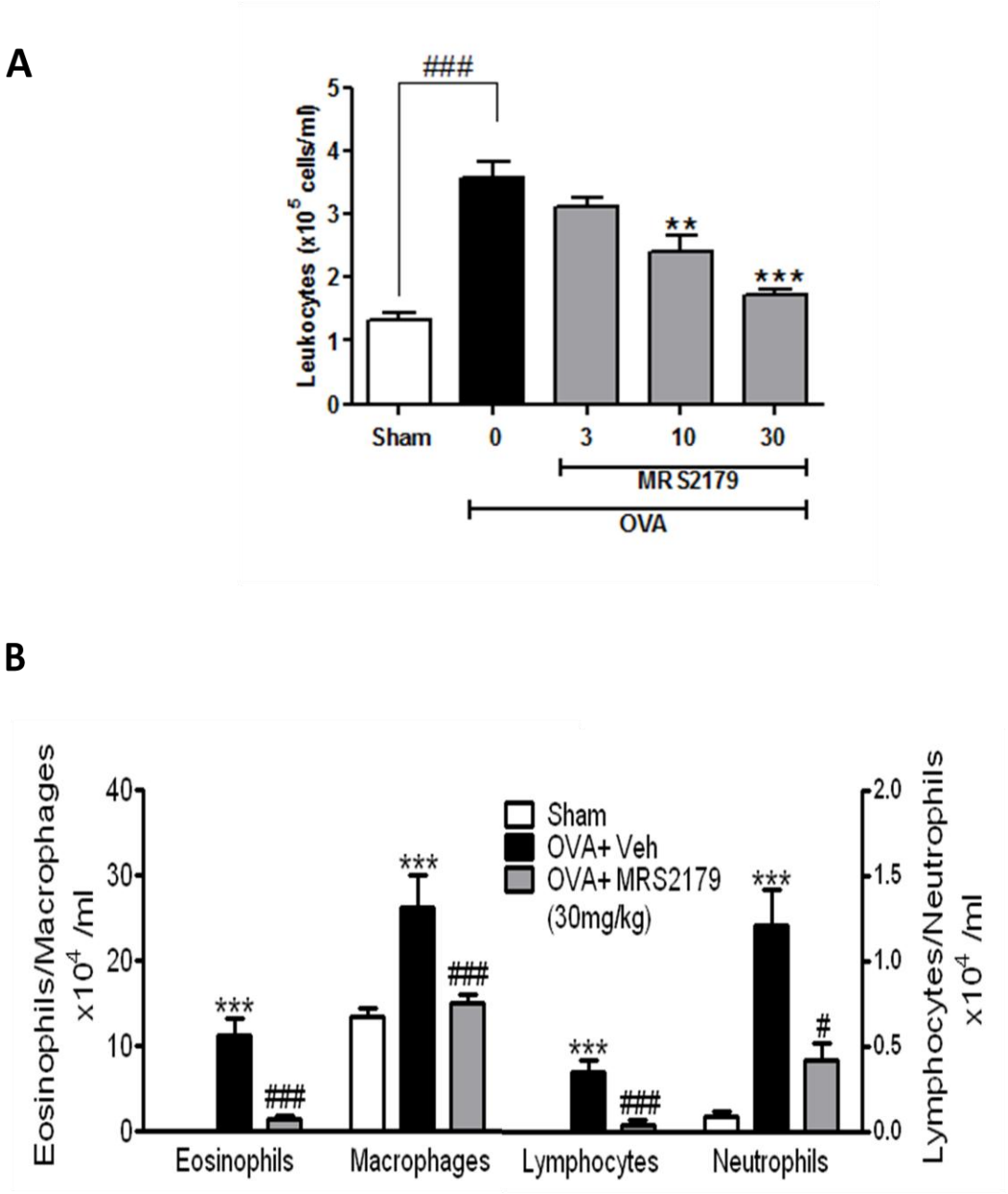


Figure 4. 2 The Effects of MRS2179 of Pulmonary Leukocyte Recruitment

Mice were subjected to a two week sensitisation (1.5mg/kg OVA) and allergen exposure protocol. MRS2179 was administered prior to allergen exposure on days 14, 15 and 16 (3, 10 & 30mg/kg intravenously). Lavage fluid was collected 24 hours post final allergen exposure (day 17). **A** Total BAL Leukocytes **B** Differential Cell counts. Data: Mean ± SEM, N= 4 animals per group. * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$ compared to sham control, # = $P < 0.05$, ## = < 0.01, ### = $P < 0.001$ compared to OVA challenged mice.

4.2.1.2 An Investigation into the Effects of the $P2Y_1$ Receptor Antagonist MRS2500 on Pulmonary Leukocyte Recruitment

Given the ability of MRS2179 to significantly inhibit leukocyte recruitment, a second more potent antagonist was used to confirm the phenomenon. MRS2500 [2-Iodo-N6-methyl-(N)-methanocarba-2'-deoxyadenosine-3',5'-biphosphate], the combination of the ring-constrained (N)-methanocarbo modification of adenine nucleotides combined with an substitution of the 2-position functionalities with a 2-iodo substitution significantly increased the antagonistic potency (Kim et al., 2003). OVA sensitised mice were pre-treated with either vehicle or the ADP analogue 0.1, 1 or 3mg/kg MRS2500 30 minutes prior to allergen challenge. Twenty-four hours post final challenge all animals were culled and BAL fluid collected.

Sensitisation and subsequent challenge to OVA resulted in significant elevation in total leukocyte lung recruitment (Sham: $1.81 \pm 0.32 \times 10^5$ cells/ml vs. OVA: $4.23 \pm 0.24 \times 10^5$ cells/ml, $P < 0.001$), treatment with MRS2500 as demonstrated, induced a dose-dependent inhibition of leukocyte recruitment (OVA: $4.23 \pm 0.24 \times 10^4$ vs. 3mg/kg MRS2500: $2.25 \pm 0.15 \times 10^4$ cells/ml, $P < 0.05$) (**Figure 4.3A**). Similarly to MRS2179, Intravenous dosing of MRS2500 at 3mg/kg inhibited allergen induced BAL differential cell leukocyte recruitment compared to vehicle controls as seen in macrophage counts (Sham: $16.00 \pm 2.79 \times 10^4$ cells/ml vs OVA: $22.80 \pm 1.65 \times 10^4$ cells/ml; 3mg/kg MRS2500: $17.96 \pm 1.99 \times 10^4$ cells/ml), neutrophil counts (Sham: 0.00 ± 0.00 cells/ml vs. OVA: $0.80 \pm 0.21 \times 10^4$ cells/ml, $P < 0.05$; 3mg/kg MRS2500: $0.08 \pm 0.08 \times 10^4$ cells/ml, $P < 0.05$), lymphocyte counts (Sham: 0.00 ± 0.00 cells/ml vs OVA: $2.00 \pm 0.38 \times 10^4$ cells/ml, $P < 0.01$; 3mg/kg MRS2500: $0.56 \pm 0.33 \times 10^4$ cells/ml, $P < 0.05$) and eosinophil counts in the lavage (Sham: 0.00 ± 0.00 cells/ml vs. OVA: $16.23 \pm 2.45 \times 10^4$ cells/ml, $P < 0.01$; 3mg/kg MRS2500: $3.92 \pm$

$3.04x10^4$ cells/ml, $P < 0.01$) (**Figure 4.3B**). Through the use of MRS2179 and MRS2500 this data heavily implicates the $P2Y_1$ receptor in the modulation of pulmonary leukocyte recruitment following allergen challenge in this murine model.

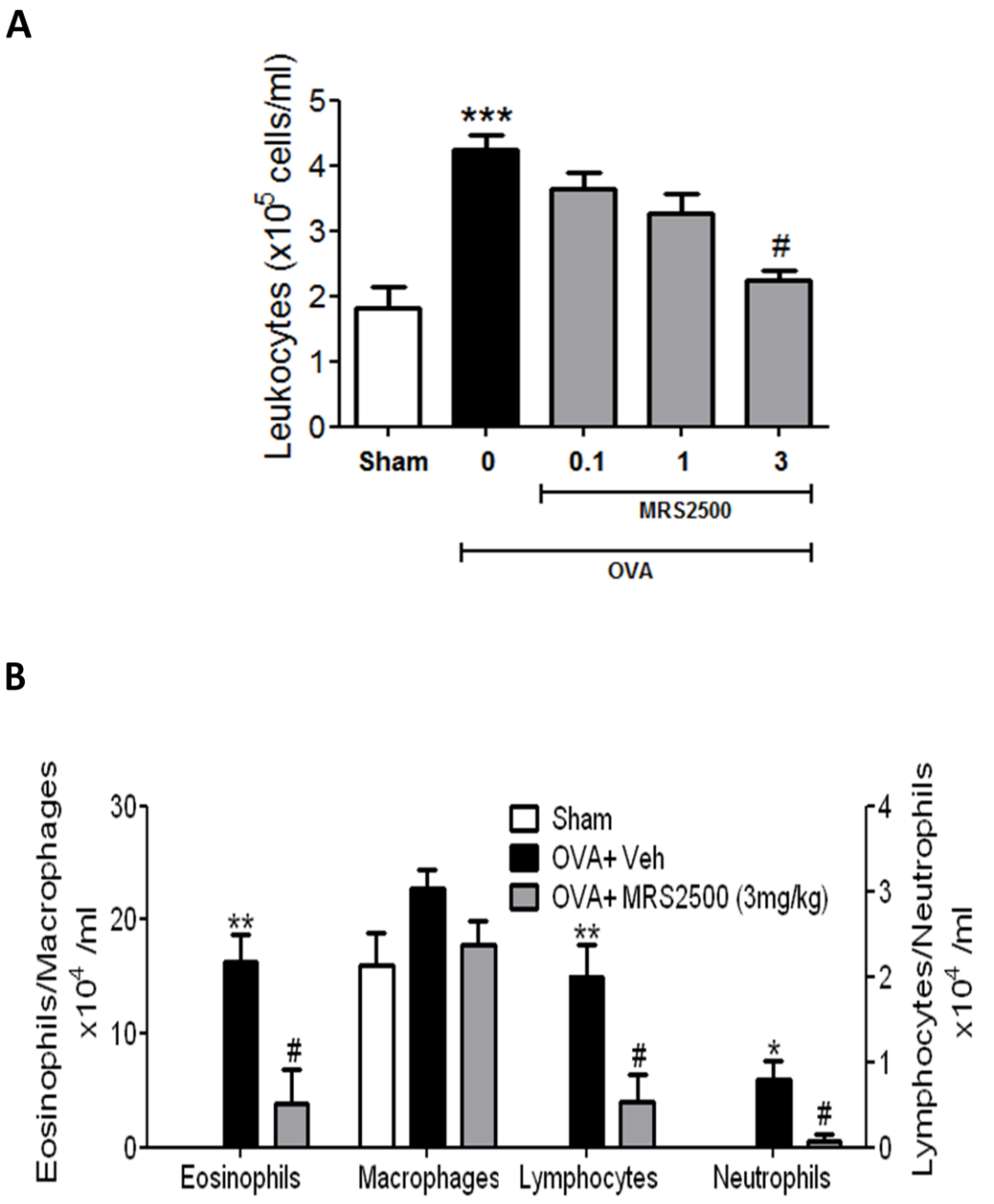


Figure 4. 3 The Effects of MRS2500 on Pulmonary Leukocyte Recruitment

Mice were subjected to a two week sensitisation (1.5mg/kg OVA) and allergen exposure protocol. MRS2500 was administered prior to allergen exposure on days 14, 15 and 16 (0.1, 1 & 3mg/kg intravenously). Lavage fluid was collected 24 hours post final allergen exposure (day 17).). **A** Total BAL Leukocytes **B** Differential Cell counts. Data: Mean ± SEM, N= 4 animals per group. * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$ compared to sham control, # = $P < 0.05$, ## = < 0.01, ### = $P <$ 0.001 compared to OVA challenged mice.

4.3.2. An Investigation into the effect of $P2Y_{12}$ Antagonism on Pulmonary Leukocyte Recruitment in a Murine Model of Allergic Inflammation

4.3.2.1. An Investigation into the Effect of the $P2Y_{12}$ Specific Receptor Antagonist MRS2395 on Pulmonary Leukocyte Recruitment *in vivo*

The ADP acyclic analogue MRS2395 which is a highly selective and competitive antagonist for the $P2Y_{12}$ receptor was dosed intravenously on days 14, 15 and 16 30 minutes prior to allergen challenge. Twenty-four hours post final challenge (Day 17) all animals were culled and BAL fluid collected.

Similarly to the studies performed with the $P2Y_1$ receptor antagonists MRS2179 and MRS2500, sensitisation and subsequent challenge to nebulised 3% OVA induced significant total leukocyte recruitment to the lungs compared to sham sensitised samples (Sham: 15.35 ± 1.52 $x10^4$ cells/ml vs. OVA: 34.00 ± 1.19$x10^4$ cells/ml, $P<0.001$), this allergen induced increase in total leukocyte recruitment was unaffected by any tested dose (1, 3 or 10mg/kg) of the $P2Y_{12}$ receptor antagonist MRS2395 (Veh: 34.00 ± 1.19$x10^4$ cells/ml vs. 10mg/kg [top dose] MRS2395: 41.75 ± 4.16$x10^4$ cells/ml) (**Figure 4.4A**). This allergen induced rise in total leukocyte recruitment was mirrored by an allergen induced rise in pulmonary differential leukocyte numbers with MRS2395 treatment failing to modulate any of either macrophage counts (Sham: 18.07 ± 3.14$x10^4$ cells/ml vs. OVA: 20.98 ± 4.07$x10^4$ cells/ml; 10mg/kg [top dose] MRS2395: 21.20 ± 1.81$x10^4$ cells/ml), neutrophil counts (Sham: 0.00 ± 0.00 cells/ml vs. 1.53 ± 0.51$x10^4$ cells/ml; 10mg/kg [top dose] MRS2395: 2.36 ± 0.49$x10^4$ cells/ml), lymphocyte counts (Sham: 0.00 ± 0.00 cells/ml vs. OVA: 1.53 ± 0.51$x10^4$ cells/ml, 10mg/kg [top dose] MRS2395: 2.36 ± 0.49$x10^4$ cells/ml) or eosinophil counts within the lavage (Sham: 0.00 ± 0.00

cells/ml vs. OVA: 9.35 ± 2.58x10^4 cells/ml, $P < 0.001$; 10mg/kg MRS2395: 14.00 ± 1.52x10^4 cells/ml) (**Figure 4.4B**).

Doses of MRS2395 were selected from previous *in* vivo based publications, however to ensure that the observed phenomenon was accurate and not an issue of the efficacy of the antagonist used, the experiment was repeated using an additional second class of $P2Y_{12}$ receptor antagonists, AR-C66096.

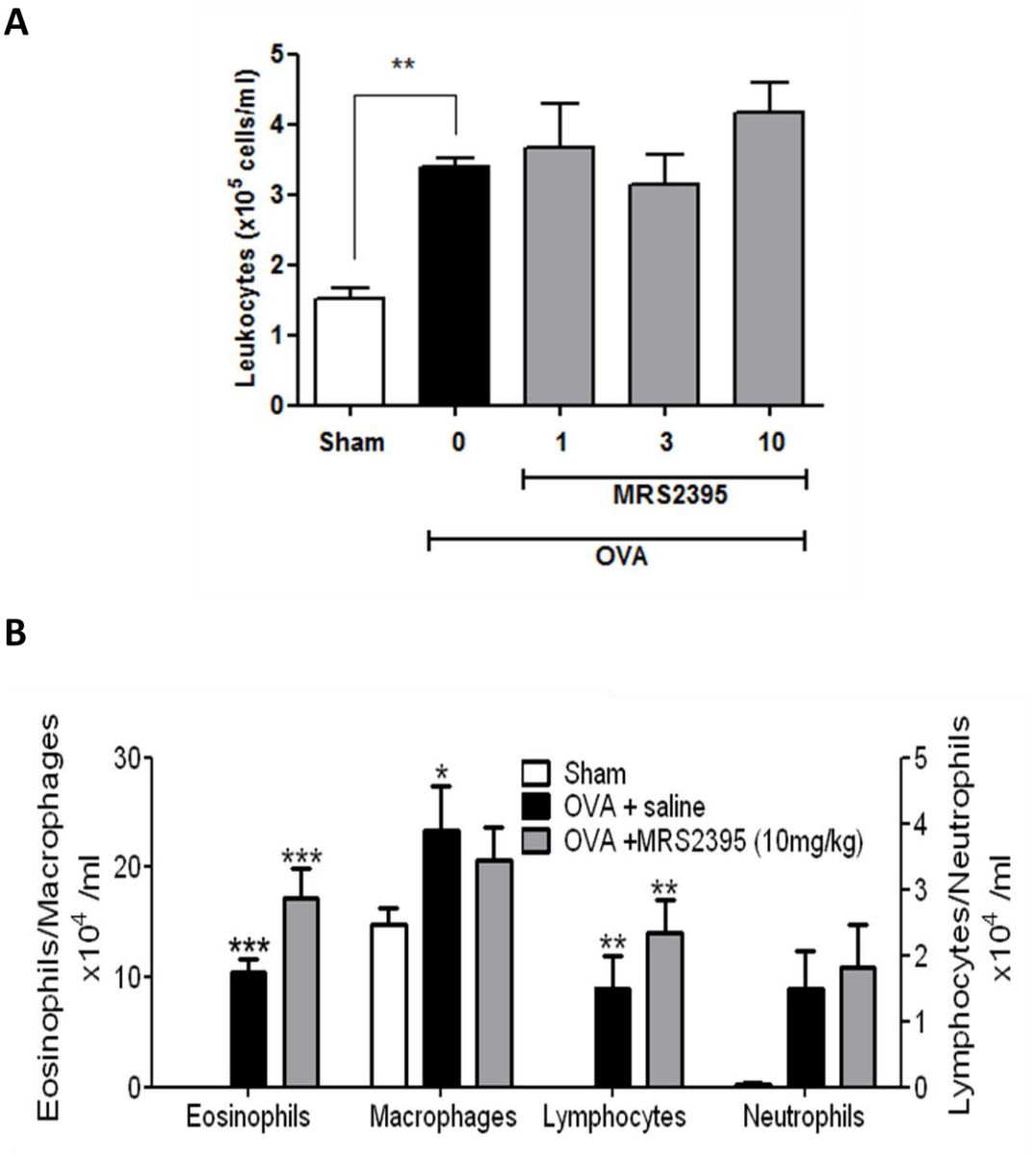


Figure 4. 4 The Effects of MRS2395 on Pulmonary Leukocyte Recruitment

.Mice were subjected to a two week sensitisation (1.5mg/kg OVA) and allergen exposure protocol. MRS2395 was administered prior to allergen exposure on days 14, 15 and 16 (0.1, 1 & 3mg/kg intravenously). Lavage fluid was collected 24 hours post final allergen exposure (day 17). **A** Total BAL Leukocytes **B** Differential Cell counts. Data: Mean ± SEM, N= 4 animals per group. * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$ compared to sham controls.

4.3.2.2 An Investigation into the Effect of the $P2Y_{12}$ Specific Receptor Antagonist AR-C66096 on Pulmonary Leukocyte Recruitment *in vivo*

In addition to the adenosine diphosphate acrylic analogue MRS2395, the adenosine triphosphate analogue AR-C66096, which also demonstrates highly selective antagonist properties for the $P2Y_{12}$ receptor, was administered to mice 30 minutes prior to allergen challenge at 0.1, 1 and 3 mg/kg. Twenty-four hours post final challenge all animals were culled and BAL fluid collected.

Sensitisation and subsequent challenge to OVA induced a significant elevation in total leukocyte pulmonary recruitment, which remained significantly elevated in all animals treated with AR-C66096 (Sham: 12.13 ± 2.12x10^4 cells/ml vs. OVA: 136.00 ± 13.68x10^4 cells/ml, P<0.001; 3mg/kg AR-C66096: 126.75 ± 21.11x10^4 cells/ml) (**Figure 4.5A**). This elevation of total cell counts in response to allergen as well as the failure of AR-C66096 to inhibit this pulmonary recruitment was reproduced in all inflammatory differential cell counts including macrophages (Sham: 12.13 ± 2.12x10^4 cells/ml vs. OVA: 52.05 ± 18.48x10^4 cells/ml, P < 0.001, 3mg/kg AR-C66096: 49.55 ± 18.48x10^4 cells/ml), neutrophils (Sham: 0.00 ± 0.00 cells/ml vs. OVA: 2.96 ± 1.60x10^4 cells/ml; 3mg/kg AR-C66096: 1.52 ± 0.70x10^4 cells/ml), lymphocytes (Sham: 0.00 ± 0.00 vs. OVA: 5.10 ± 1.28x10^4 cells/ml; 3mg/kg AR-C66096: 7.50 ±.51x10^4 cells/ml, P < 0.05) and eosinophils (Sham: 0.00 ± 0.00 cells/ml vs. OVA: 69.41 ±18.96x10^4 cells/ml, P < 0.001; 3mg/kg AR-C66096: 68.18 ± 23.92x10^4 cells/ml) (**Figure 4.5B**).

Additionally, experiments performed by collaborators at the Institute of Internal and Vascular Medicine at the University of Perugia in Italy demonstrated a failure of 75mg/kg Clopidogrel (the $P2Y_{12}$ specific thienopyridine antagonist) to inhibit allergen induced total leukocyte (Sham: 0.73 ± 0.06x10^5 cells/ml vs. OVA: 3.89 ±

$0.18x10^5$ cells/ml, $P < 0.001$; 75mg/kg Clopidogrel: $3.69 \pm 0.28x10^5$ cells/ml, **Figure 4.6A**) and eosinophil (Sham: $0.04 \pm 0.04x10^5$ cells/ml vs. OVA: $2.29 \pm 0.07x10^5$ cells/ml, $P < 0.001$; 75mg/kg Clopidogrel: $1.77 \pm 0.12x10^5$ cells/ml, **FIgure 4.6B**) recruitment in the same murine model of allergic inflammation.

Using the 3 distinct $P2Y_{12}$ receptor antagonists MRS2395, AR-C66096 and Clopidogrel, there is substantial evidence indicating a failure of $P2Y_{12}$ receptor signalling to modulate allergen induced pulmonary leukocyte recruitment within this murine model of allergic inflammation, in stark contrast to the results obtained through the use of the $P2Y_1$ receptor antagonists MRS2179 and MRS2500 which significantly attenuate allergen induced pulmonary leukocyte recruitment.

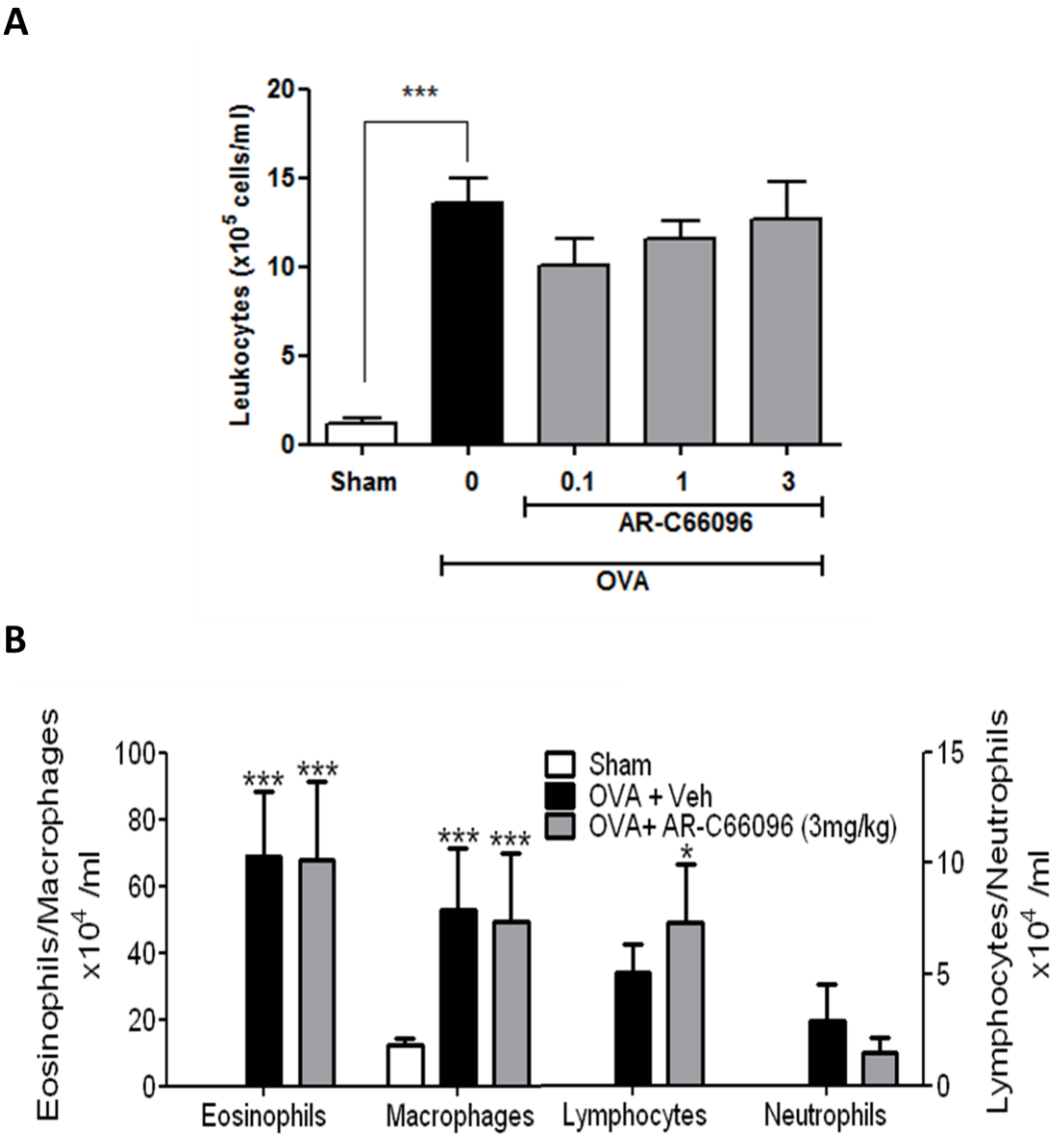


Figure 4. 5 The Effects of AR-C66096 on Pulmonary Leukocyte Recruitment

Mice were subjected to a two week sensitisation (1.5mg/kg OVA) and allergen exposure protocol. AR-C66096 was administered prior to allergen exposure on days 14, 15 and 16 (0.1, 1 & 3mg/kg intravenously). Lavage fluid was collected 24 hours post final allergen exposure (day 17). **A** Total BAL Leukocytes **B** Differential Cell counts. Data: Mean ± SEM, N= 4 animals per group. * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$ compared to sham controls.

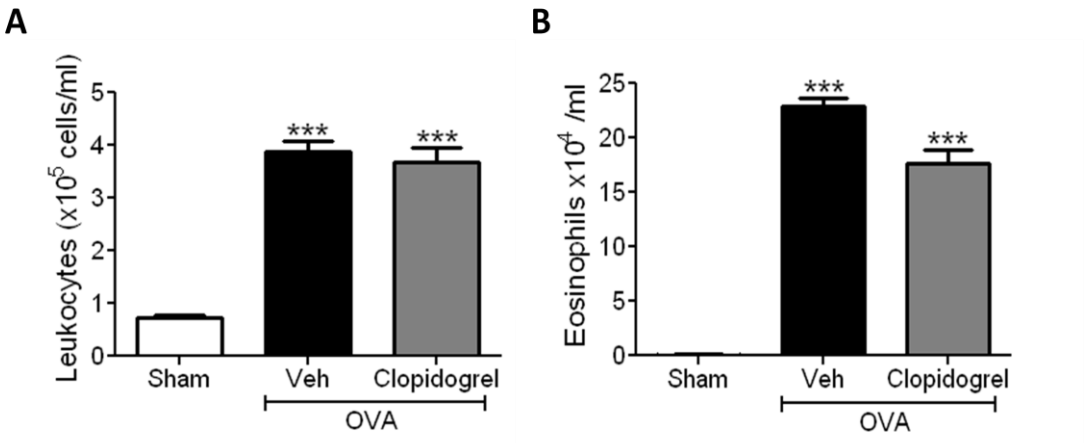


Figure 4. 6 The effects of Clopidogrel of Pulmonary Leukocyte Recruitment

Mice were subjected to a two week sensitisation (1.5mg/kg OVA) and allergen exposure protocol. 75mg/kg Clopidogrel was administered *i.v.* prior to allergen exposure on days 14, 15 and 16. Lavage fluid was collected 24 hours post final allergen exposure (day 17). **A** Total BAL Leukocytes **B** Differential Cell counts. Data: Mean ± SEM, N= 4 animals per group. *** = $P < 0.001$ compared to sham controls.

4.4 An Investigation into the Effect of the $P2X_1$ Receptor Antagonist NF-279 on Pulmonary Leukocyte Recruitment in a Murine Model of Allergic Inflammation

The final receptor present on the platelets surface sensitive to purine stimulation is the $P2X_1$ receptor sensitive to ATP. Whilst this receptor is not sensitive to ADP it has previously been shown to be involved in platelet shape change and Ca^{2+} mobilisation, two processes involved in the initial stages of platelet activation (Sun et al., 1998; Toth-Zsamboki et al., 2003). The involvement of this receptor in allergen induced pulmonary leukocyte recruitment was subsequently evaluated using the $P2X_1$ suramin analogue NF-279.

OVA sensitised mice were pre-treated with intravenous vehicle or 0.1, 1 or 3mg/kg NF-279, 30 minutes prior to 3% nebulised OVA exposure on days 14, 15 and 16.

Twenty-four hours post final challenge (Day 17) all animals were culled and BAL fluid collected.

Sensitisation and challenge to nebulised OVA induced a significant elevation in total pulmonary leukocyte numbers compared to sham sensitised mice which was unaffected following treatment of NF-279 at all tested doses (Sham: 12.00 ± 2.29x10^4 cells/ml vs. OVA: 33.3 ± 4.41x10^4 cells/ml, $P < 0.001$; 3mg/kg NF-279: 38.50 ± 2.86x10^4 cells/ml) (**Figure 4.7A**). This allergen induced increase in pulmonary leukocyte recruitment, combined with the persistent elevation of leukocyte recruitment following treatment with all doses of NF-279 was also replicated within the differential cells present in the lungs including macrophages (Sham: 11.92 ± 0.32x10^4 cells/ml vs. OVA: 22.86 ± 5.14x10^4 cells/ml; 3mg/kg NF-279: 19.43 ± 10.07x10^4 cells/ml), neutrophils (Sham: 0.06 ± 0.03x10^4 cells/ml vs. OVA: 0.94 ± 0.46x10^4 cells/ml, 3mg/kg NF-279: 1.31 ± 0.74x10^4 cells/ml, $P < 0.05$), lymphocytes (Sham: 0.00 ± 0.00 cells/ml vs. OVA: 0.43 ± 0.10x10^4 cells/ml; 3mg/kg NF-279: 0.36 ± 0.20x10^4 cells/ml, $P < 0.05$) and eosinophils (Sham: 0.00 ± 0.00 cells/ml vs. OVA: 5.77 ± 0.26x10^4 cells/ml, $P < 0.001$; 3mg/kg NF-279: 4.56 ± 0.30x10^4 cells/ml, $P < 0.001$) (**Figure 4.7B).**

Experiments with antagonists for the $P2Y_1$, $P2Y_{12}$ and $P2X_1$ receptors suggests that the $P2Y_1$ receptors but not the $P2Y_{12}$ or $P2X_1$ receptors are important in the induction of pulmonary leukocyte recruitment in OVA sensitised mice following provocation to nebulised OVA.

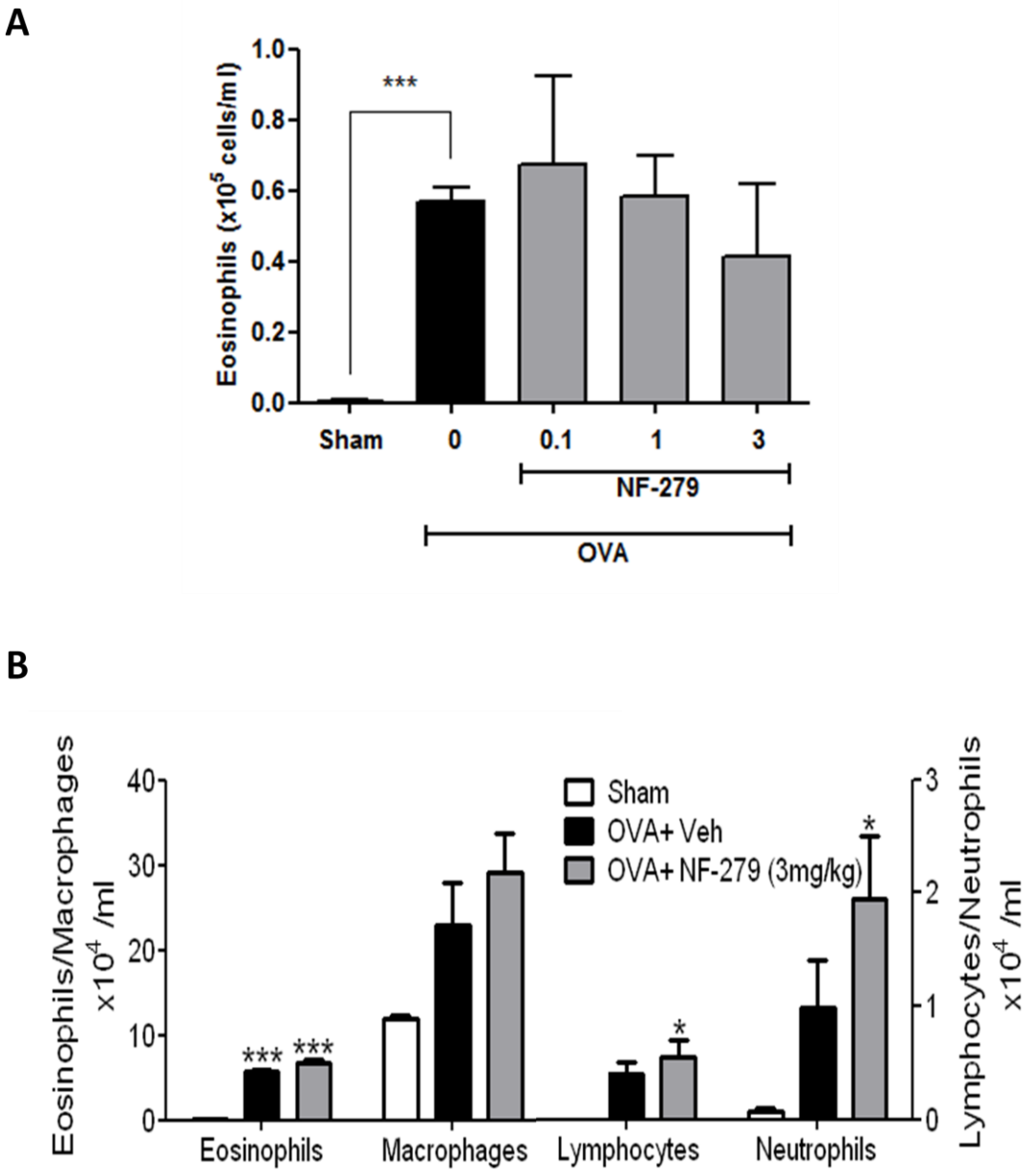


Figure 4. 7 The Effects of NF-279 on Pulmonary Leukocyte Recruitment

Mice were subjected to a two week sensitisation (1.5mg/kg OVA) and allergen exposure protocol. NF-279 was administered prior to allergen exposure on days 14, 15 and 16 (0.1, 1 & 3mg/kg intravenously). Lavage fluid was collected 24 hours post final allergen exposure (day 17). **A** Total BAL Leukocytes **B** Differential Cell counts. Data: Mean ± SEM, N= 4 animals per group. * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$ compared to sham controls.

4.5 An Investigation into the Purinergic Modulation of Platelet Aggregation

The inability of platelet $P2Y_{12}$ receptor blockade to modulate platelet-dependent leukocyte recruitment demonstrated a clear difference in function from the $P2Y_1$ receptor; however it was important to investigate if the absence of an effect through the $P2Y_{12}$ receptor was a result of poor efficacy or inadequate doses of both MRS2395 and AR-C66096. Therefore the activity of both the $P2Y_1$ and $P2Y_{12}$ receptor antagonists were tested using an *ex vivo* assay of platelet aggregometry. Female Balb/c mice were sensitised to OVA using the previously described sensitisation protocol and 30 minutes before challenge, mice were then treated with either 3mg/kg MRS2500 (**Figure 4.8A & 4.8B**), 3mg/kg AR-C66096 (**Figure 4.8C**) or 75mg/kg Clopidogrel as a positive control (**Figure 4.8D**). Platelets ($2x10^8$ plts/ml) were isolated from murine blood 6 hours post allergen challenge and platelet aggregation towards 2-10μM ADP measured *ex vivo*.

Platelet aggregation towards 2-10μM ADP was unaltered following sensitisation and exposure to allergen compared to sham controls (Sham: 50.82 ±4.36% vs. OVA: 55.91 ± 4.31%) (**Figure 4.8**). Platelet samples pre-treated with 3mg/kg of the $P2Y_1$ receptor antagonist MRS2500 inhibited platelet aggregation at 1hr post treatment (Vehicle: 79.93 ± 6.62% vs. 3mg/kg MRS2500: 47.77 ± 4.71%, $P < 0.05$) but not 6 hours post challenge (Veh: 52.50 ± 7.50% vs. 3mg/kg MRS2500: 40.75 ± 4.01%) (**Figure 4.8A & 4.8B**).

Platelets taken from mice administered with either 3mg/kg AR-C66096 (Veh: 57.85 ± 5.54% vs. AR-C66096: 31.29 ± 6.98%, $P<0.05$, (**Figure 4.8C**) or 75mg/kg Clopidogrel (Veh: 50.00 ± 12.60% vs. 75mg/kg Clopidogrel: 14.00 ± 2.10% $P<0.05$, **Figure 4.8D**) demonstrated significant suppression of ADP induced aggregation,

confirming their activity in this murine model of inflammation. This is critical in being able to conclude that pulmonary leukocyte recruitment was $P2Y_{12}$ insensitive in this murine model of allergic inflammation.

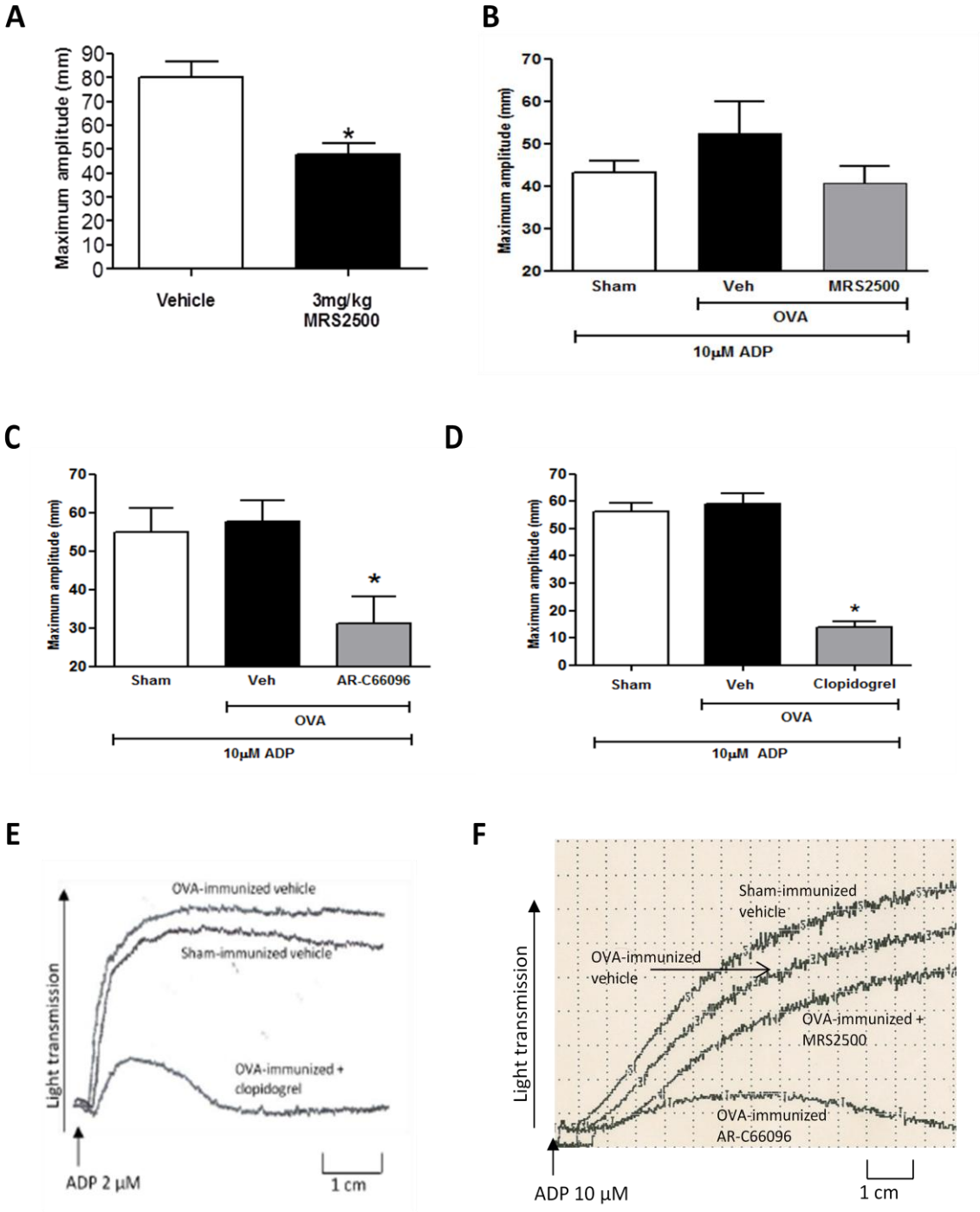


Figure 4. 8 The Effects of $P2Y_1$ and $P2Y_{12}$ receptor antagonism on platelet aggregation

Mice were subjected to a two week sensitisation (1.5mg/kg OVA) and single allergen exposure protocol. Prior to allergen exposure, mice were treated with either 3mg/kg MRS2500 at 1hr (**A**), 3mg/kg MRS2500 at 6hrs (**B**), 3mg/kg AR-C66096 at 6hrs (**C**) or vehicle control and 75mg/kg Clopidogrel at 6 hrs (**D**) Platelets were collected 6 hours later and aggregation monitored to 10μM ADP. **E**/**F** Example traces of purinergic antagonism of ADP induced platelet aggregation. Data: Mean ± SEM, N= 4 animals per group. * = $P < 0.05$.

4.6 An Investigation into the Effects of $P2Y_1$ and $P2Y_{12}$ Receptor Antagonism in Platelet-Depleted Mice

Through the use of the specific $P2Y_1$ (MRS2179, MRS2500) and $P2Y_{12}$ (MRS2395, AR-C66096) receptor antagonists the $P2Y_1$ receptor was identified to be the only platelet expressed purinergic receptor involved in pulmonary leukocyte recruitment as no suppression of allergen induced leukocyte recruitment was observed following treatment with $P2Y_{12}$ or $P2X_1$ receptor antagonists.

$P2Y_1$ receptor expression however is not solely located to the platelets surface with systemic expression detected in epithelial, endothelial, kidney and leukocyte subtypes (Burnstock *et al* 2007). It is therefore conceivable that the phenomenon of leukocyte recruitment was the result of leukocyte activation by ADP and not solely as a result of platelet activation. Therefore using a model of platelet depletion and subsequent reinfusion of washed platelets, the importance of platelet specific $P2Y_1$ receptors in allergen induced pulmonary leukocyte recruitment was investigated.

4.6.1 An Investigation into the Effects of Busulfan Treatment on Circulating Platelet Levels

Using the murine model of allergic inflammation as previously described, a significant elevation in total leukocyte numbers as well as macrophage, neutrophils, lymphocytes and eosinophil numbers ($P<0.001$) detected in the lungs was observed compared to sham controls.

Pre-treatment of mice with a dosing regimen of 25mg/kg busulfan (days -5, -3, 0), a bone marrow depleting toxin (figure 2.3) induced a severe state of thrombocytopenia compared to vehicle treated control mice (Veh: $4.22 \pm 0.31 \times 10^8$ platelets/ml vs. Busulfan: $1.18 \pm 0.03 \times 10^9$ platelets/ml, $P<0.001$) thus demonstrating a decrease in

circulating platelet numbers of 64% (**figure 4.9**). Whilst busulfan treatment rendered mice thrombocytopenic, no effect on circulating leukocyte numbers, including mononuclear cells, neutrophils or eosinophils was induced by busulfan treatment (**Figure 4.9**); suggesting that busulfan administration at this dose specifically depleted platelets.

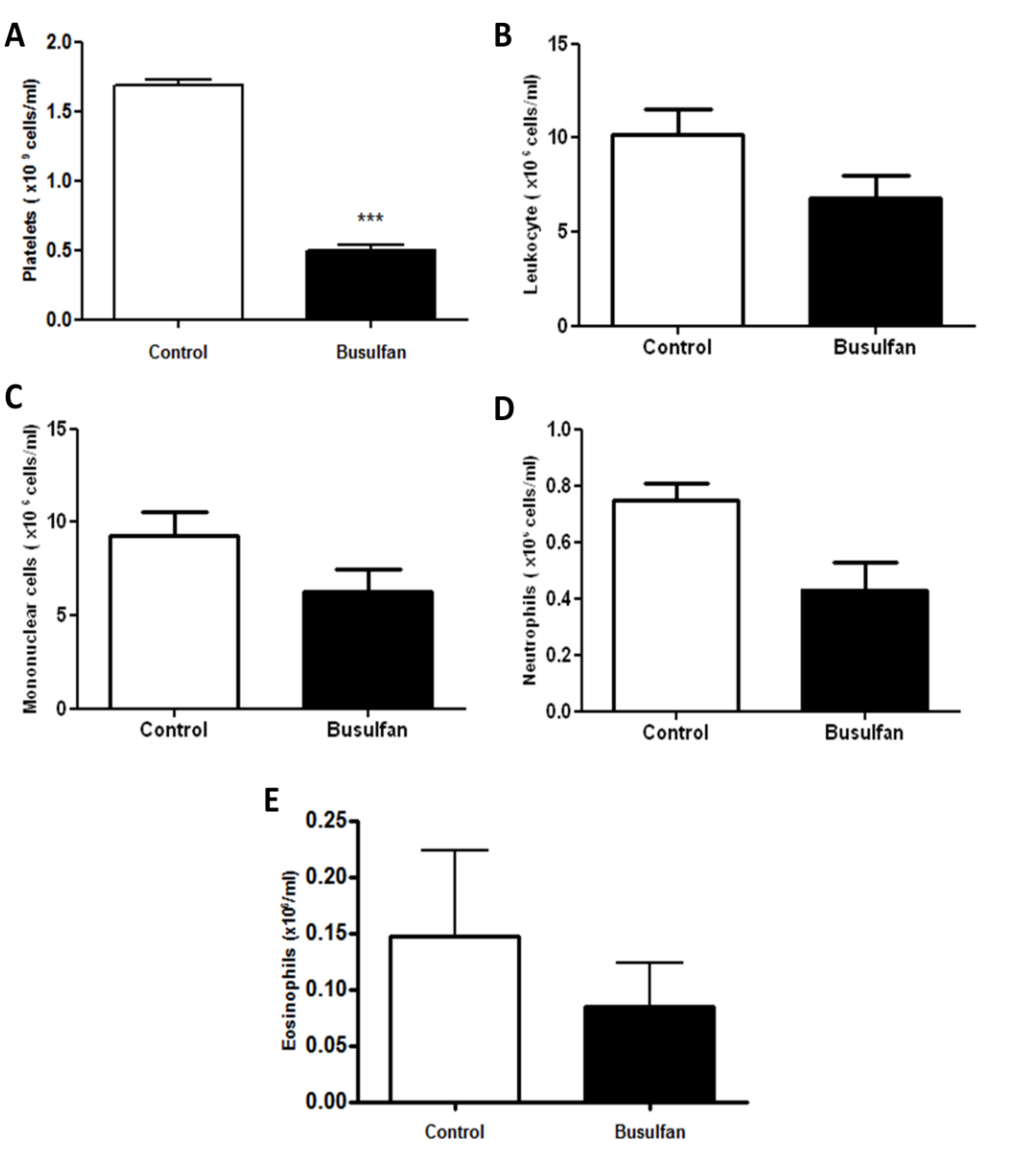


Figure 4. 9 Effects of Busulfan Treatment On Circulating Platelet and Leukocyte Numbers

Mice were treated with busulfan (25mg.kg ip) on days 0, 3, and 5 and blood was collected via cardiac bleed on day 19. BAL and analysed for platelets (A), total cell counts (B), mononuclear cells (C), neutrophils (D), and eosinophils (E), lymphocytes (C), macrophages (D), and neutrophils (E). n=4. Data are expressed as mean± SEM. *** $P < 0.001$.

4.6.3 An investigation into the Role of Platelets on Pulmonary Leukocyte Recruitment *in vivo*

Mice rendered thrombocytopenic through Busulfan treatment demonstrated significantly reduced total leukocyte recruitment to the lungs of allergen challenged mice compared to OVA sensitised controls (Sham: 13.92 ± 1.56x104 cells/ml; Veh: 103.77 ± 17.91x10^4 cells/ml vs. Busulfan: 37.35 ± 7.91x10^5 cells/ml, P<0.05) (**Figure 4.10A**). This attenuation of allergen induced total leukocyte recruitment was replicated in all the differential cell counts including macrophages (Sham: 13.80 ± 1.54x10^4 cells/ml; Vehicle: 35.28 ± 6.25x10^4 cells/ml vs. Busulfan: 15.19 ± 2.62x10^4 cells/ml, P < 0.01, **figure 4.10B**), lymphocytes (Sham: 0.01 ± 0.01x10^4 cells/ml; Vehicle: 2.94 ± 0.79x10^4 cells/ml vs. Busulfan: 0.67 ± 0.20x10^4 cells/ml, P < 0.05, **figure 4.10C**), neutrophils (Sham: 0.19 ± 0.08x10^4 cells/ml; Vehicle: 1.03 ± 0.46x10^4 cells/ml vs. Busulfan: 0.28 ± 0.18x10^4 cells/ml, **figure 4.10D**) and eosinophils (Sham: 0.00 ± 0.00 cells/ml; Vehicle:71.05 ± 12.31x10^4 cells/ml vs. Busulfan: 18.78 ± 5.55x10^4 cells/ml, P < 0.05, **figure 4.10E**)

Busulfan induced suppression of pulmonary total leukocyte recruitment was restored through the reinfusion of washed platelets (WP) from donor mice also sensitised to allergen (Busulfan: 37.35 ± 7.91x10^4 cells/ml vs. WP: 82.95 ± 18.75x10^4 cells/ml, **figure 4.10A**), a process that was replicated in all cell types: macrophages (Busulfan: 15.19 ± 2.62x10^4 cells/ml vs. WP: 27.48 ± 4.87x10^4 cells/ml, **figure 4.10B**), lymphocytes (Busulfan: 0.67 ± 0.20x10^4 cells/ml vs. WP: 2.26 ± 0.50x10^4 cells/ml, **figure 4.10C**), neutrophils (Busulfan: 0.28 ± 0.18x10^4 cells/ml vs. WP: 1.20 ± 0.86x10^4 cells/ml, **figure 4.10D**) and eosinophils (Busulfan: 18.78 ± 5.54 vs. WP: 59.91 ± 16.70, **figure 4.10E**) confirming previous work demonstrating the critical role of platelets in pulmonary leukocyte recruitment as described previously (Pitchford et al., 2003a, 2005).

4.6.4 An Investigation into the Impact of Platelet Specific $P2Y_1$ Receptors on Pulmonary Leukocyte Recruitment

On days 14 and 15 of the immunisation and challenge protocol for the current model of allergic inflammation, platelets were isolated from donor mice sensitised to OVA and subsequently treated *in vitro* with concentrations in excess of those required to inhibit platelet aggregation (100μM) of either MRS2500 or AR-C66096. After incubating for 15 minutes at room temperature any free unbound drug was removed from the platelet suspension through centrifugation before being reinfused into thrombocytopenic mice at a concentration of $1.40x10^8$ platelets/mouse before allergen challenge on days 14 and 15.

As previously stated, reinfusion of WP into thrombocytopenic mice restored allergen induced leukocyte recruitment which was abolished following busulfan treatment. A significant attenuation remained with platelets treated with 100μM of the $P2Y_1$ antagonist MRS2500 of total leukocyte recruitment (OVA: 103.77 ± $17.91x10^4$ cells/ml vs. MRS2500: 41.15 ± $9.82x10^4$ cells/ml, $P<0.05$, **Figure 4.10A**) as well as all differential cell types: macrophages (OVA: 35.28 ±$6.25x10^4$ cells/ml vs MRS2500: 18.71 ± 3.14 $x10^4$ cells/ml, $P < 0.01$, **figure 4.10B**), lymphocytes (OVA: 2.94 ± $0.79x10^4$ cells/ml vs. MRS2500: 0.69 ± $0.32x10^4$ cells/ml, $P < 0.05$, **figure 4.10C**), neutrophils (OVA: 1.03 ± $0.46x10^4$ cells/ml vs. MRS2500: 0.09 ± $0.05x10^4$ cells/ml, **figure 4.10D**) and eosinophils (OVA: 71.05 ± $12.31x10^4$ cells/ml vs. MRS2500: 21.22 ± $8.20x10^4$ cells/ml, $P < 0.05$, **figure 4.10E**).

In contrast however, reinfusion with platelets treated with saturating concentrations of AR-C66096 failed to inhibit total leukocyte recruitment when compared to OVA challenged mice with normal circulating platelet levels (OVA: 103.77 ± $17.91x10^4$ cells/ml vs. AR-C66096: 84.95 ± $19.37x10^4$ cells/ml, **figure 4.10A**) and also failed

to inhibit the recruitment of the additional differential cell numbers; macrophages (OVA: 103.77 ± 17.91x10^4 cells/ml vs. AR-C66096: 84.95 ± 19.38x10^4 cells/ml, **figure 4.10B**), lymphocytes (OVA: 2.94 ± 0.79x10^4 cells/ml vs. AR-C66096: 1.92 ± 0.69x10^4 cells/ml, **figure 4.10C**), neutrophils (OVA: 1.03 ± 0.46x10^4 cells/ml vs. AR-C66096: 1.37 ± 0.64x10^4 cells/ml, **figure 4.10D**) and eosinophils (OVA: 71.05 ± 12.32x10^4 cells/ml vs. AR-C66096: 56.64 ± 16.97x10^4 cells/ml, **figure 4.10E**). This confirms that the modulation of pulmonary leukocyte recruitment via $P2Y_1$ receptors in the earlier studies was mediated specifically by platelet expressed $P2Y_1$ receptors and not systemically expressed $P2Y_1$ receptors.

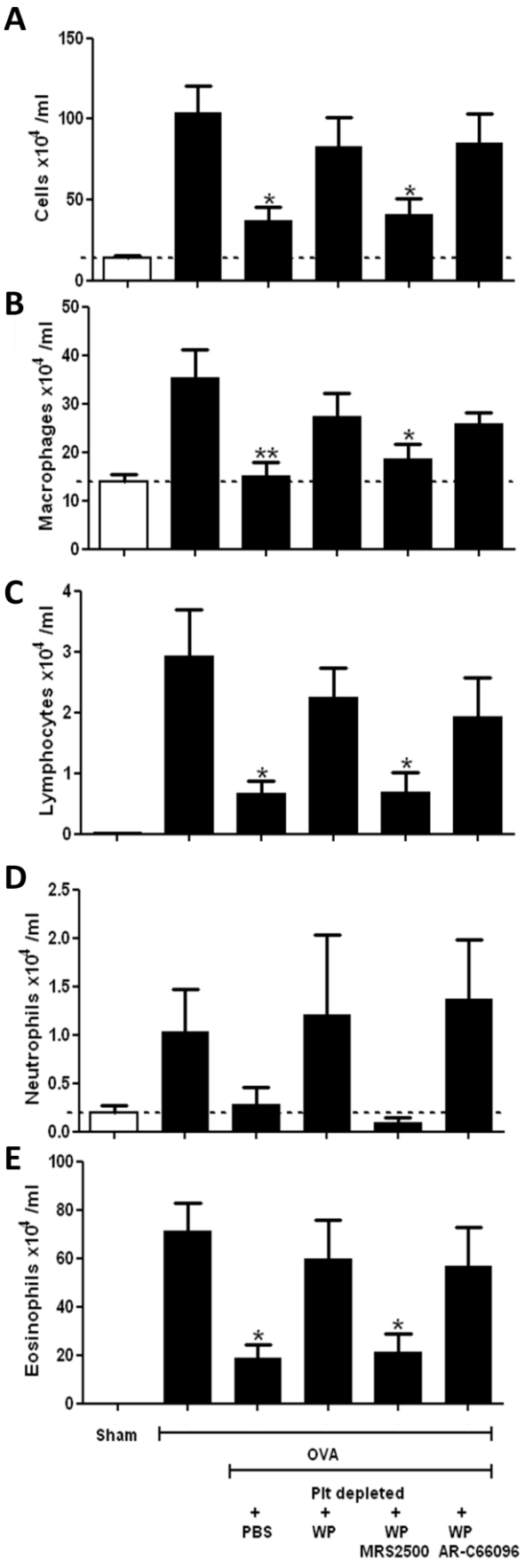


Figure 4. 10 The Effects of MRS2500 and AR-C66096 treated Platelets on Pulmonary Leukocyte Recruitment

Mice were rendered thrombocytopenic through a dosing protocol involving the bone marrow depleting agent Busulfan. During this process mice were subjected to a two week sensitisation (1.5mg/kg OVA) and allergen exposure protocol. Washed platelets isolated from allergen sensitised mice were pre-treated with either vehicle or 100µM MRS2500 or AR-C66096. Treated platelets were subsequently reinfused in thrombocytopenic mice prior to allergen exposure on days 14 and 15. Lavage fluid was collected 24 hours post final allergen exposure (day 17). **A** Total BAL Leukocytes **B** Macrophages **C** Lymphocytes **D** Neutrophils **E** Eosinophils. Data: Mean ± SEM, N= 10-12 animals per group. * = $P < 0.05$, ** = $P < 0.01$ compared to sham controls.

4.7 An Investigation into the Role of Purinergic Receptors in Adhesion Molecule Expression and Platelet-Leukocyte Interactions

4.7.1. An Investigation into the Role of the $P2Y_1$ and $P2Y_{12}$ Receptors on ADP Induced P-selectin Expression 6 hours Post Allergen Challenge

P-selectin is an adhesion molecule stored within the platelet α-granules. Upon platelet activation, these α-granules fuse with the platelet membrane secreting its contents. This triggers an upregulation of surface expressed P-selectin. This allows the quantification of surface P-selectin expression to be used as a marker of platelet activation.

Using the *in vitro* assay of P-selectin expression described and validated in section 3.2, ADP-induced P-selectin expression was measured in platelet populations treated with either 1, 10, 100 or 1000nM MRS2500 or AR-C66096 in platelets isolated from either sham or OVA-sensitised mice.

MRS2500 failed to inhibit ADP-induced increases in either percent surface expression of P-selectin in either sham (PBS: 45.07 ± 3.05% vs. 10μM ADP: 63.09 ± 1.11%; 1000nM MRS2500 [top dose]: 51.01 ± 6.95%) or OVA-sensitised platelets (PBS: 25.14 ± 8.15% vs. 10μM ADP: 58.63 ± 4.20%; 1000nM MRS2500 [top dose]: 20.02 ± 7.92%, **figure 4.11A**). Neither did MRS2500 demonstrate any significant inhibition on ADP-induced elevations in platelet MFI in either sham (PBS: 1.63 ± 0.08 vs. 10μM ADP: 2.43 ± 0.33, 1000nM MRS2500 [top dose]: 1.83 ± 0.16) or OVA-sensitised samples (PBS: 1.09 ± 0.23 vs. 10μM ADP: 2.05 ± 0.14; 1000nM MRS2500 [top dose]: 0.33 ± 0.19, **figure 4.11B**).

In contrast, pre-treatment of platelets with AR-C66096 demonstrated significant attenuation of the percent P-selectin expression in both sham (PBS: 24.13 ± 0.23%

vs. 10μM ADP: 60.43 ± 4.18, P < 0.01; 1000nM AR-C66096 [top dose]: 29.08 ± 5.23, P < 0.01) and OVA sensitised samples (PBS: 25.58 ± 7.30% vs. 10μM ADP: 74.59 ± 13.19%, P < 0.01; 1000nM AR-C66096 [top dose]: 54.62 ± 5.09%, P < 0.01, **figure 4.11C**). AR-C66096 treatment also inhibited ADP-induced elevations in platelet P-selectin MFI in both sham (PBS:1.00 ± 0.09 vs. 10μM ADP: 2.14 ± 0.23, P < 0.05; 1000nM AR-C66096 [top dose]: 1.11 ± 0.15, P < 0.05) and OVA-sensitised samples (PBS: 1.09 ± 0.23 vs. 10μM ADP: 2.05 ± 0.14, P < 0.05; 1000nM AR-C66096: 0.86 ± 0.19, P < 0.01, **figure 4.11D**).

Sensitisation to allergen had no impact on ADP induced elevations in percent platelet P-selectin expression (Sham-sensitised: 61.76 ± 2.03% vs. OVA-sensitised: 66.61 ± 7.14%) when compared to unstimulated controls (Sham-sensitised: 36.60 ± 6.17% vs. OVA-sensitised: 25.36 ± 4.47%) allergen sensitisation also had no effect on ADP-induced changes in MFI on platelet P-selectin samples (Sham-sensitised: 2.29 ± 0.19 vs. OVA-sensitised: 2.05 ± 0.09) when compared to unstimulated controls (Sham-sensitised: 1.32 ± 0.19 vs. OVA-sensitised: 1.09 ± 0.13). It is possible that this apparent failure of the $P2Y_1$ receptor to inhibit allergen induced P-selectin expression 6 hours post allergen challenge is an artifact of the platelet isolation procedure. Basal levels of P-selectin expression of nearly 40% suggests that the platelet is already activated decreasing the therapeutic window of the $P2Y_1$ receptor antagonists.

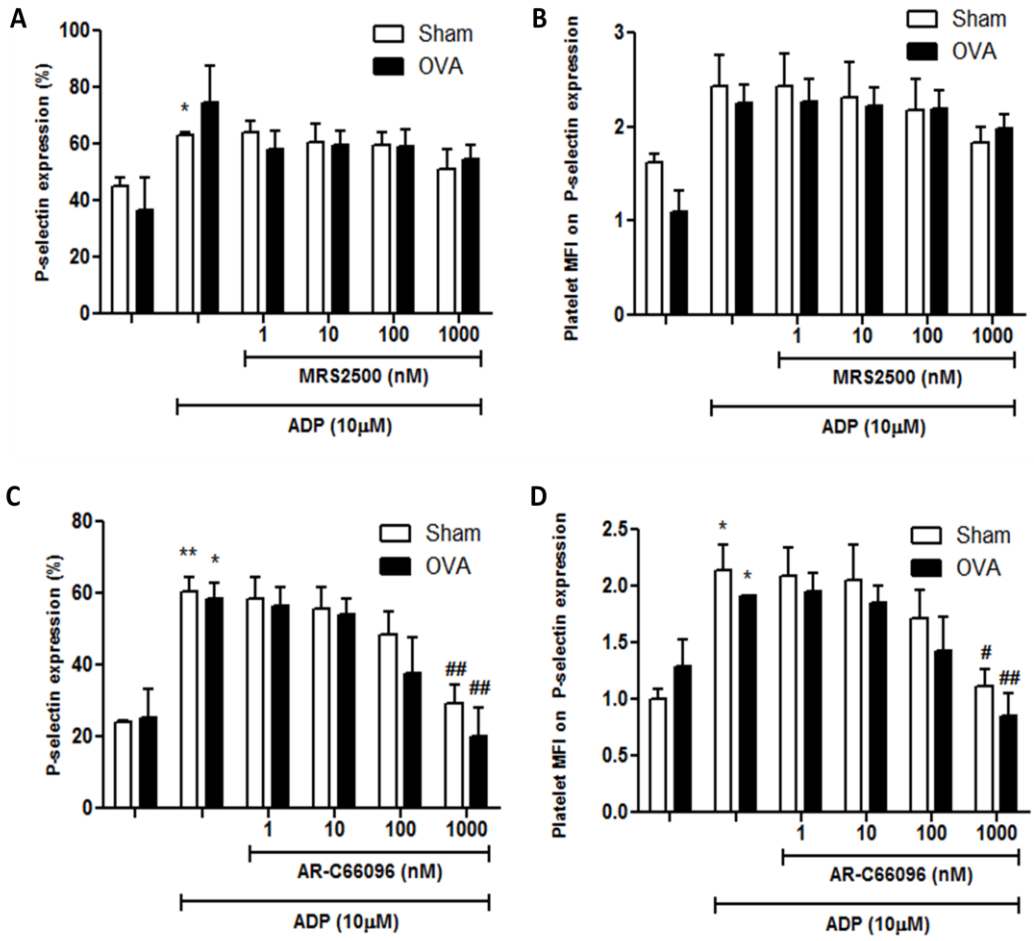


Figure 4. 11 The Effects of MRS2500 and AR-C66096 on ADP Induced Platelet P-selectin *in vitro*

Platelets were isolated from blood of mice either sham or 1.5mg/kg OVA sensitised. Platelets were treated with 1, 10, 100 or 1000nM MRS2500 or AR-C66096 and P-selectin expression measured following stimulation with 10μM ADP. Data expressed as mean ± SEM, n=3. * = $P < 0.05$, ** = $P < 0.01$, # = $P < 0.05$, ## = $P < 0.01$.

4.7.2 An Investigation into the Role of the $P2Y_1$ and $P2Y_{12}$ Receptors on ADP Induced Platelet-Leukocyte Conjugation 6 hours Post Allergen Challenge

Platelet P-selectin is a necessary component of membrane interactions between platelets and leukocytes in allergic inflammation and is believed to be critical in the formation of circulating platelet-leukocyte complexes which are suspected to be responsible for the 'priming' of inflammatory leukocytes, a process suspected to be required for efficient leukocyte adhesion to endothelial cells. Indeed, a number of studies have shown elevated levels of circulating platelet-leukocyte complexes both in asthmatic patients following allergen challenge, and in a murine model of allergic inflammation, known to be P-selectin-dependent (Pitchford *et al*. 2003; 2005).

Given the inconclusive data produced through the measurement of platelet surface P-selectin expression, the occurrence of these platelet-leukocyte complexes were used as an alternate marker of platelet activation following the administration of either the $P2Y_1$ (MRS2500) or $P2Y_{12}$ (AR-C66096) receptor antagonists at 6 and 24 hours post allergen challenge. A significant increase in circulating platelet-leukocyte complexes (Sham: 18.73 ± 2.61% vs. OVA: 35.75 ± 4.30, $P<0.01$ **figure 4.12**), and the relative number of platelets (CD41 MFI) attached per individual leukocyte observed (Sham: 0.92 ± 0.08% vs. OVA: 1.85 ± 0.35%, $P<0.05$ **figure 4.12**) was measured in allergen-sensitised mice over sham controls 6 hours post allergen challenge.

OVA-sensitised mice pre-treated with 3mg/kg MRS2500 failed to show any increase in the percent of circulating platelet-leukocyte conjugates at 6 hours post allergen challenge (Sham: 18.73 ± 2.61% vs. MRS2500: 28.15 ± 2.64%, **figure 4.12A**), neither did MRS2500 treated mice demonstrate a significant increase in the MFI on platelet-leukocyte complexes (Sham: 0.92 ± 0.08 vs. MRS2500: 1.23 ± 0.12, **figure 4.12B**). In contrast, OVA-sensitised mice treated with 3mg/kg AR-C66096

demonstrated a significant elevation in both the percent of circulating platelet-leukocyte complexes (Sham: 18.73 ± 2.61% vs. AR-C66096: 32.21 ± 3.22%, $P < 0.05$, **figure 4.12A**) and platelet MFI on platelet-leukocyte complexes (Sham: 0.92 ± 0.08 vs. AR-C66096: 1.76 ± 0.29, $P < 0.05$, **figure 4.12B**) at 6 hours post allergen challenge.

By 24 hours post allergen challenge, the significant elevation in the percent of circulating platelet-leukocyte complexes (Sham: 24.38 ± 2.30% vs. OVA: 29.54 ± 1.30%, **figure 4.12C**) or platelet MFI on platelet-leukocyte complexes (Sham: 1.46 ± 0.17 vs. OVA: 1.80 ± 0.20, **figure 4.12D**) had returned to levels similar to sham sensitised mice. Furthermore no significant differences were noted in mice pre-treated with either 3mg/kg MRS2500 or 3mg/kg AR-C66096 compared sham levels in either the percent of circulating platelet-leukocyte complexes (Sham: 23.38 ± 2.30% vs. MRS2500: 29.54 ± 1.30% or AR-C66096: 27.79 ± 1.62%, **figure 4.12C**) or platelet MFI on platelet-leukocyte complexes (Sham: 1.46 ± 0.17 vs. MRS2500: 1.63 ± 0.16 or AR-C66096: 1.62 ± 0.17, **figure 4.12D**).

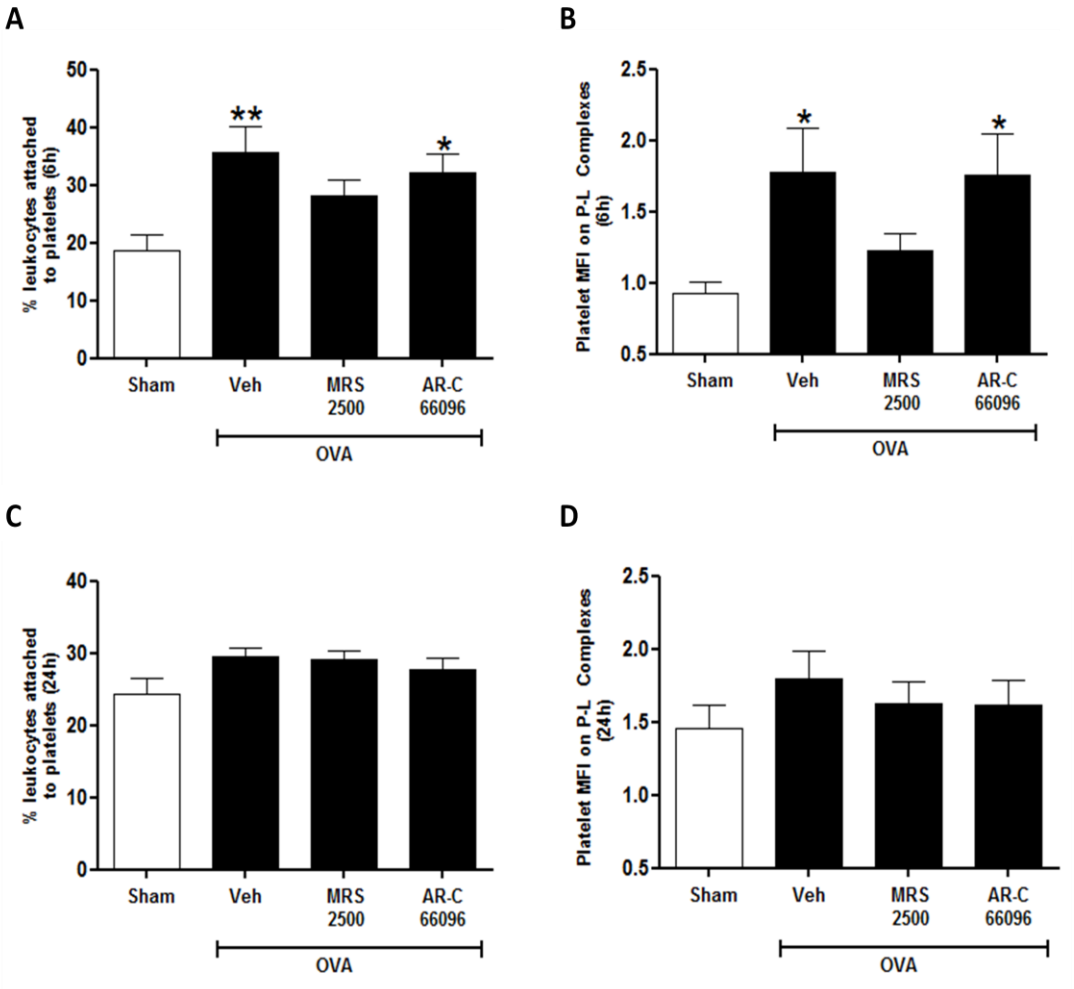


Figure 4. 12 The Effects of MRS2500 and AR-C66096 on the Formation of Circulating Platelet-Leukocyte Conjugates 6 and 24 Hours Post Allergen Challenge

Mice were subjected to a two week sensitisation (1.5mg/kg OVA) and single allergen exposure protocol. Prior to allergen exposure, mice were treated with vehicle control, 3mg/kg MRS2500 or 3mg/kg AR-C66096. Whole blood was isolated at 6 (**A** & **B**) and 24 (**C** & **D**) hours later and circulating platelet-leukocyte conjugates measured by flow cytometry. Data expressed as either % circulating conjugates (**A** & **C**) or MFI (**B** & **D**). Data: Mean ± SEM, N= 6-8 animals per group. * = $P < 0.05$, ** = $P < 0.01$ compared to sham controls.

4.7.3 An Investigation into the Impact of Purinergic Receptor Antagonism on Circulating Platelet and Leukocyte Numbers

Extensive research shows that stimulation of both the $P2Y_1$ and $P2Y_{12}$ receptors are capable of inducing P-selectin expression (Leon *et al.* 2003, 2004; *Straub et al.* 2011), Thus it was surprising to observe a $P2Y_1$ specific mediation of platelet-leukocyte aggregate formation. It was therefore important to see if the suppression of platelet-leukocyte aggregate formation through treatment with the $P2Y_1$ antagonist MRS2500 was a result of thrombocytopenia or changes in haematopoiesis.

Circulating platelet numbers demonstrated a modest thrombocytopenia (albeit insignificant) in OVA-sensitised animals compared to sham controls (Sham: 10.77 ± $1.76x10^8$ platelets/ml vs. OVA: 8.89 ± $0.83x10^8$ platelets/ml) (**Figure 4.13A**). Platelet numbers were also unaffected following either $P2Y_1$ or $P2Y_{12}$ pre-treatment (MRS2500: 11.48± 1.27 $x10^8$ platelets/ml; AR-C66096: 9.43± $0.42x10^8$ platelets/ml). Similarly to circulating platelet numbers, circulating leukocyte numbers were also measured. Allergen sensitisation failed to modulate leukocyte numbers compared to sham controls (Sham: 4.80 ± 1.73 $x10^6$ cells/ml vs. OVA: 7.80 ± 1.31 $x10^6$ cells/ml) (**Figure 4.13B**). Circulating leukocyte numbers also remained unaffected following pre-treatment MRS2500 or AR-C66096 (MRS2500: 6.30 ± $1.51x10^6$ cells/ml; AR-C66096: 7.09 ± $1.97x10^6$ cells/ml).

Given that treatment with the purinergic agents MRS2500 and AR-C66096 failed to alter circulating platelet or leukocyte numbers in allergen-challenged mice the observed suppression of platelet-leukocyte complex formation by MRS2500 was not a result of thrombocytopenia or any changes to haematopoiesis.

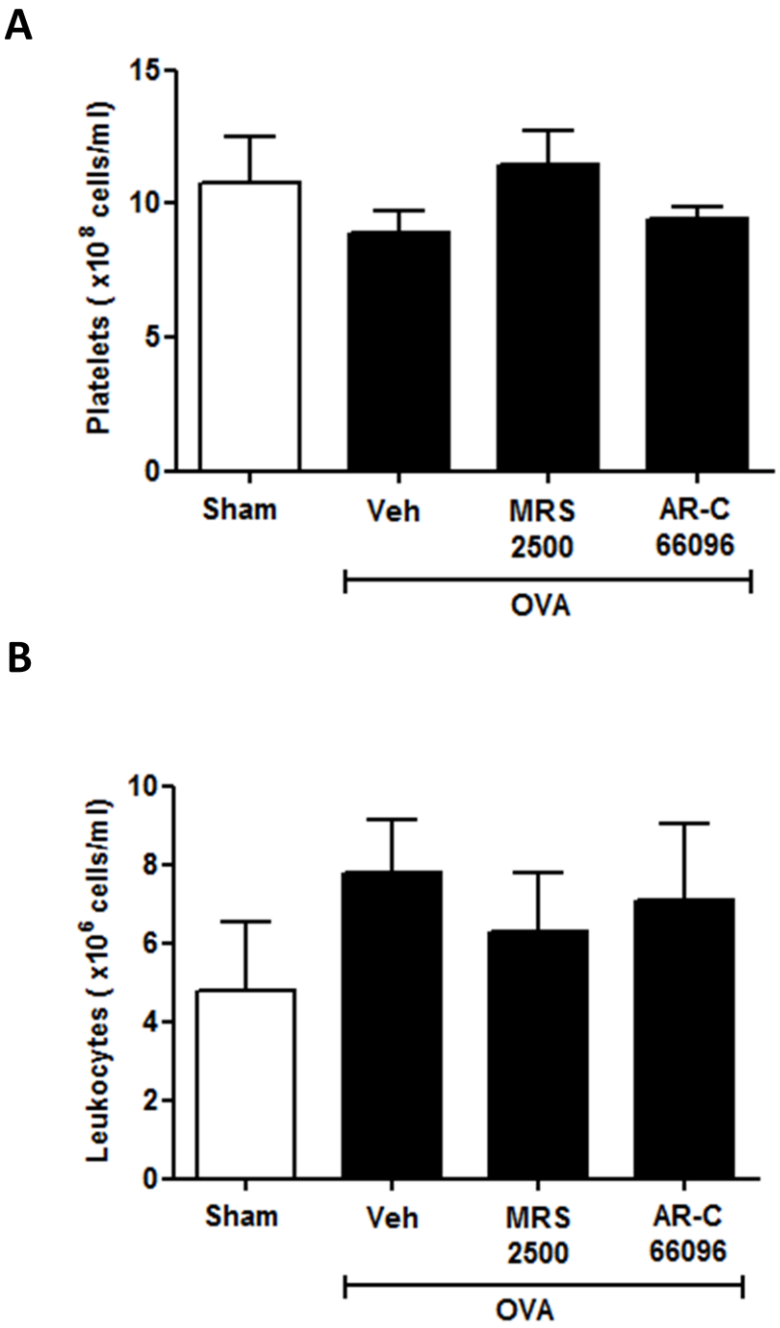


Figure 4. 13 The Effects of OVA Sensitisation and MRS2500/AR-C66096 Administration On Circulating Platelet and Leukocyte Numbers

Mice sensitised to 1.5mg/kg OVA were treated with 3mg/kg MRS2500 or AR-C66096 and subsequently challenged to 3% aerosolised OVA on day 14. Blood was taken at 6 hours post allergen challenge and subsequently circulating platelet and leukocyte numbers quantified using a Zeiss axioscope microscope under a x20 objective. Data: Mean ± SEM, N=6-11 animals per group.

4.7.4 An Investigation into the Effects of Allergen Sensitisation on Platelet Surface Expression of $P2Y_1$ or $P2Y_{12}$ Receptors

After the discovery that purinergic receptor treatment had no effect on circulating platelet or leukocyte numbers, the possibility of altered surface expression of platelet $P2Y_1$ or $P2Y_{12}$ was considered, as a possible cause of the $P2Y_1$ specific suppression of platelet-leukocyte complex formation through elevated levels of $P2Y_1$ expression in comparison to $P2Y_{12}$ receptor expression. Expression of surface $P2Y_1$ and $P2Y_{12}$ was measured by flow cytometry with representative scatter plots of P2Y expression on CD41 stained platelets in **figure 4.14.**

$P2Y_1$ receptor expression remained unaffected by allergen sensitisation and challenge compared to sham controls (Sham: 9.38 ± 0.64% vs. OVA: 9.19 ± 0.89%) (**Figure 4.15A**), this was replicated by the relative number of receptors expressed per individual receptors (Sham: 1.00 ± 0.04% vs. OVA: 1.03 ± 0.03%) (**Figure 4.15**), culminating in a binding index of 0.095 ± 0.01 for sham against 0.095 ± 0.012 for OVA sensitised samples (**Figure 4.15C**). Similar to that of $P2Y_1$ receptor expression, both the percentage expression of $P2Y_{12}$ receptors (Sham: 45.90 ± 1.19% vs. OVA: 42.91 ± 1.74%) (**Figure 4.16A**) and the relative number of $P2Y_{12}$ receptors expressed per individual platelet (Sham: 2.85 ± 0.11% vs. OVA: 2.73 ± 0.08%) (**Figure 4.16B**) were unaffected by allergen sensitisation, demonstrating a binding index of 1.312 ± 0.08 for sham controls against 1.174 ± 0.081in OVA sensitised platelets (**Figure 4.16C**), thus indicating that the suppression of platelet-leukocyte conjugation by MRS2500 but not AR-C66096 treatment was not a result of altered surface expression of $P2Y_1$ or $P2Y_{12}$ receptors on platelets.

Figure 4.17 depicts a single band for the ab74996 anti-$P2Y_1$ antibody approximately 56-58kDa and a single band for the anti-$P2Y_{12}$ antibody ab82725 at approximately 39kDa thus confirming the specificity of the antibodies for the $P2Y_1$ and $P2Y_{12}$ receptors respectively.

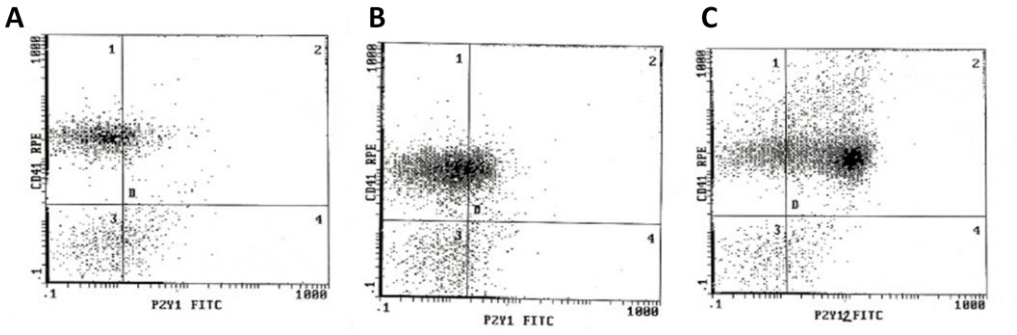


Figure 4. 14 The Effects of OVA Sensitisation on Platelet $P2Y_1$ and $P2Y_{12}$ Receptor Expression

Mice sensitised to 1.5mg/kg OVA were treated with 3mg/kg MRS2500 or AR-C66096 and subsequently challenged to 3% aerosolised OVA on day 14. Blood was taken 6 hours post allergen challenge and platelet $P2Y_1$ and $P2Y_{12}$ receptor expression was assessed by flow cytometry. Representative scatter plots are shown: (**A**) anti-CD41-FITC + control IgG-PE, (**B**) anti-CD41-FITC + anti-$P2Y_1$-PE, (**C**) anti-CD41-FITC + anti-$P2Y_{12}$-PE.

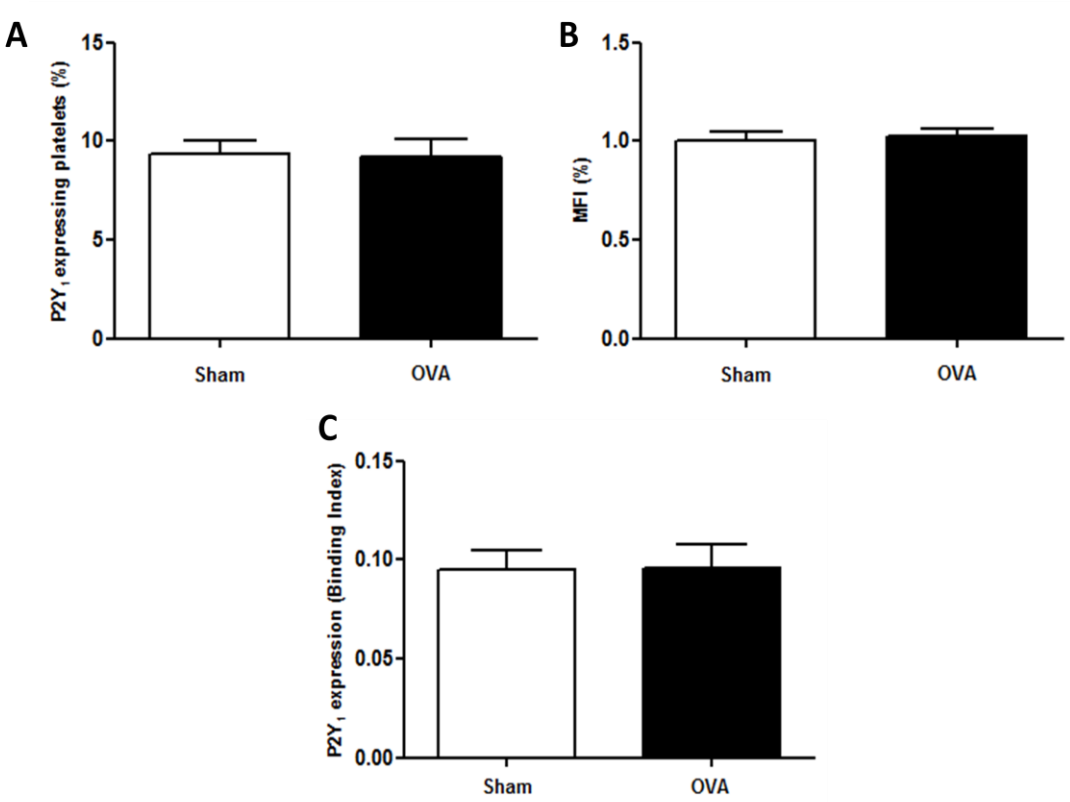


Figure 4. 15 The Effects of OVA Sensitisation on Platelet $P2Y_1$ Receptor Expression

Mice sensitised to 1.5mg/kg OVA were treated with 3mg/kg MRS2500 and subsequently challenged to 3% aerosolised OVA on day 14. Blood was taken 6 hours post allergen challenge and platelet $P2Y_1$ receptor expression was assessed by flow cytometry. The % of platelets expressing platelets (**A**) and the relative number of receptors expressed per platelet (**B**) and binding index (**C**) was quantified in platelets isolated from sham and OVA sensitised mice. Data expressed as mean ± SEM, N=4.

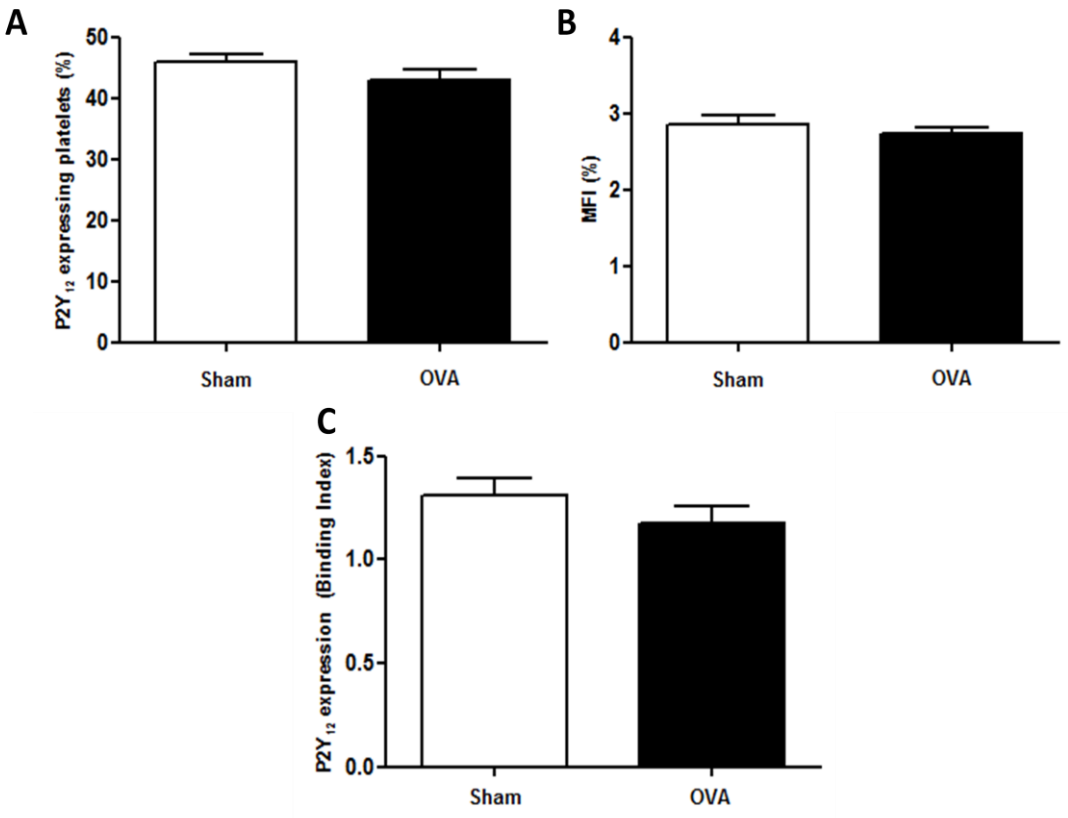


Figure 4. 16 The Effects of OVA Sensitisation on Platelet $P2Y_{12}$ Receptor Expression

Mice sensitised to 1.5mg/kg OVA were treated with 3mg/kg AR-C66096 and subsequently challenged to 3% aerosolised OVA on day 14. Blood was taken 6 hours post allergen challenge and platelet $P2Y_{12}$ receptor expression was assessed by flow cytometry. The % of platelets expressing platelets (**A**) and the relative number of receptors expressed per platelet (**B**) and binding index (C) was quantified in platelets isolated from sham and OVA sensitised mice. Data expressed as mean ± SEM, N=4.

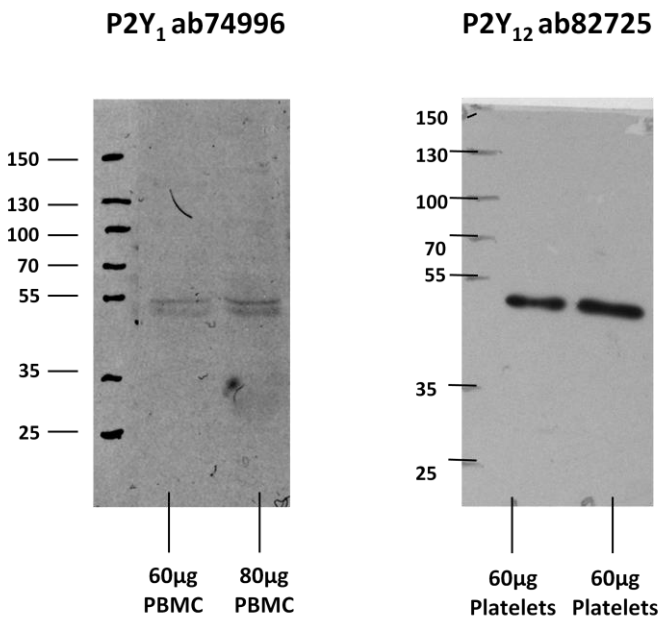


Figure 4. 17 Specificity Profile of the $P2Y_1$ and $P2Y_{12}$ Receptor Antibodies

Human derived PBMCs or Platelets were isolated and lysed with western blot analysis performed to identify the specificity of the $P2Y_1$ antibody ab74996 or the $P2Y_{12}$ antibody ab82725 for each receptor as depicted by the number of bands present.

4.8 An Investigation into the Impact of the $P2Y_1$ and $P2Y_{12}$ Receptors in Platelet-Induced Leukocyte Chemotaxis, a P-selectin Dependent Process

The formation of platelet-leukocyte complexes after allergen challenge is known to be a P-selectin dependent process (Pitchford et al., 2005), and has been shown to be affected by $P2Y_1$ signalling (**Figure 4.13**). It was hypothesised that the engagement of platelets with leukocytes *in vivo*, and in turn their dependency on $P2Y_1$ signalling may stimulate leukocyte activation and motility necessary for diapedesis from the vasculature into the tissue. Polymorphonuclear (PMN) cells have previously been shown to require the presence of platelets to undergo chemotaxis towards either MDC or TARC (Kornerup *et al* 2010). These chemokines have been heavily implicated in the asthmatic phenotype with high expression of both MDC and TARC in the lungs of human asthmatic patients (Hirata *et al*. 2003; Leung *et al* 2004).

Furthermore, both MDC and TARC have also been shown to activate platelets via the chemokine receptor CCR4 in an ADP dependent process (Kowalska *et al* 2000; Clemetson *et al* 2000; Gear *et al* 2001). It was therefore of interest to investigate whether the ability of platelets to induce the chemotaxis of bone marrow derived PMN cells towards MDC was a P-selectin dependent process and if a similar impact of $P2Y_1$ receptor modulation was observed.

Using the *in vitro* model of platelet-induced leukocyte chemotaxis validated in section 3.3, chemotaxis assays were performed to identify the importance of P-selectin in platelet-induced leukocyte migration as well as the role of the platelet $P2Y_1$ and $P2Y_{12}$ receptors.

4.8.2 An Investigation into the Role of P-selectin in Platelet-Induced PMN Cell Migration *in vitro*

Previous research has demonstrated a requirement of pulmonary leukocyte recruitment to be P-selectin dependent in a similar murine model of allergic inflammation (Pitchford et al., 2005). Furthermore extensive work has demonstrated a critical role of P-selectin in the formation of platelet-leukocyte aggregates, a process thought to be a prerequisite for platelet induced leukocyte migration. It was therefore important to confirm whether this *in vitro* model of platelet-induced PMN cell migration towards MDC was also P-selectin dependent. As such, platelets were pre-treated with a specific anti-P-selectin antibody known to block P-selectin adhesion. Pre-treatment with the anti-P-selectin antibody significantly attenuated platelet dependent PMN migration (Veh: 3.40 ± 0.42 vs. anti-P-selectin: 2.15 ± 0.38, $P<0.01$, **Figure 4.18**). This demonstrates that the requirement for platelets in the induction of PMN migration towards MDC was a P-selectin dependent phenomenon.

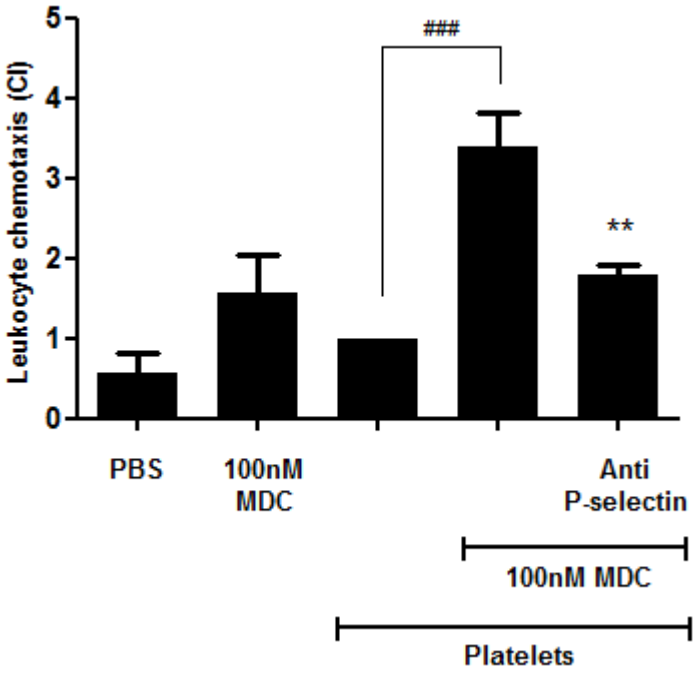


Figure 4. 18 Platelet Induced PMN chemotaxis *in vitro* is P-selectin Dependent

Platelets and PMNs were isolated from citrated blood and bone marrow from 1.5mg/kg OVA sensitised mice. Platelets were then pre-incubated with an anti-P-selectin mAb before subsequent stimulation with 100nM ADP before mixing with PMN cells. The cell mixture was added to the top well of a chemotaxis chamber. PMN chemotaxis towards MDC (bottom well 100nM) was then quantified after a 90 minute incubation and analysed as a chemotactic index. Data are expressed as mean ± SEM. ** = $P < 0.01$ where indicated to column 4, ### = $P < 0.001$ compared to column 3, n = 5.

4.8.3 An Investigation into the Impact of Allergen Sensitisation on Platelet Induced PMN Cell Migration *in vitro*

Patients with asthma demonstrate an elevation in the incidence of circulating platelet-PMN aggregates after allergen exposure (Gresele et al., 1993; Pitchford et al., 2003a; Johansson et al., 2012). Therefore, the impact of allergen-sensitisation on platelet-induced PMN chemotaxis towards MDC *in vitro* was assessed. Chemotaxis assays were prepared using platelets and PMNs isolated from blood and bone

marrow of sham- and allergen-sensitised mice. Incubation of platelets with PMNs produced significant PMN chemotaxis towards MDC compared to negative controls, whilst allergen sensitised samples demonstrated a significant elevation in PMN migration towards MDC compared to platelets isolated from sham-sensitised mice (sham: 9.06 ± 1.01 vs. OVA: 16.23 ± 1.62, P < 0.001, **Figure 4.19**).

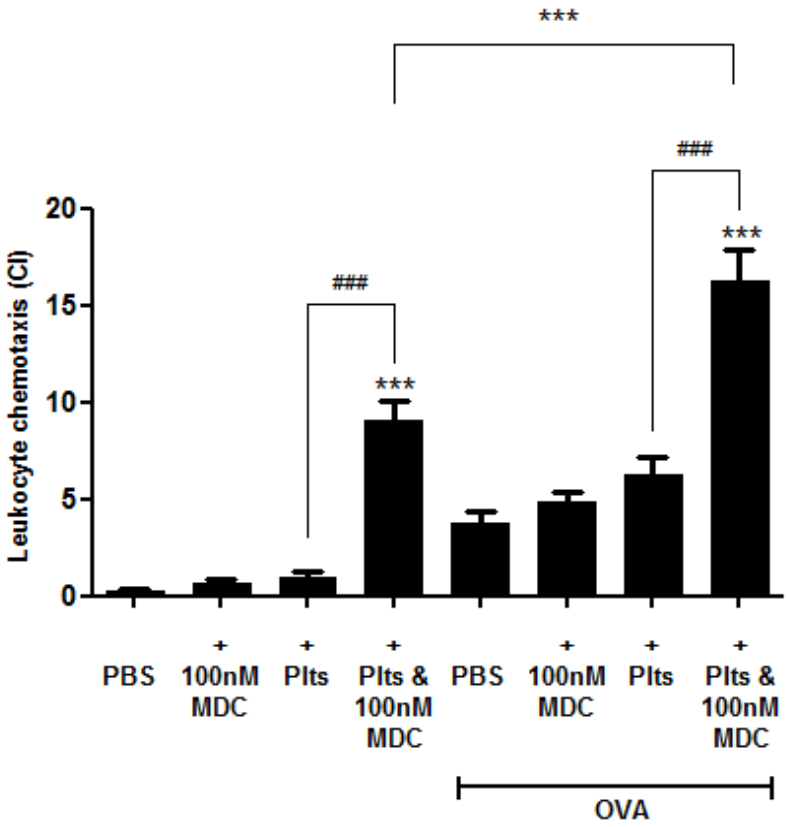


Figure 4. 19 The Effects of Allergen Sensitisation on Platelet Induced PMN Chemotaxis *in vitro*

Platelets and PMNs were isolated from citrated blood and bone marrow from either sham or 1.5mg/kg OVA sensitised mice. Platelets were then stimulated with 100nM ADP before mixing with PMN cells. The cell mixture was added to the top well of a chemotaxis chamber. PMN chemotaxis towards MDC (bottom well 100nM) was then quantified after a 90 minute incubation and analysed as a chemotactic index. Data are expressed as mean ± SEM. *** = P < 0.001 where indicated to column 4, ### = P < 0.001 where indicated to column 3 or 7,
n =4.

4.8.4 An Investigation into the Role of the $P2Y_1$ and $P2Y_{12}$ Receptors in Platelet Induced PMN Cell Migration towards MDC

Given the previous experiments demonstrating a critical role for platelet $P2Y_1$ receptors in pulmonary leukocyte recruitment in a murine model of allergic inflammation, platelets were incubated with either the $P2Y_1$ receptor antagonists MRS2179 or MRS2500, the $P2Y_{12}$ receptor antagonists MRS2395 or AR-C66096 or the $P2X_1$ receptor antagonist NF-279 prior to stimulation with 100nM ADP. Stimulated platelets were subsequently isolated from either sham or OVA sensitised mice added to PMN cells to stimulate their chemotaxis in response to 100nM MDC. In all experiments, migration was achieved only when platelets were incubated with PMN cells (**Figures 4.20 & 4.21**). Prior to stimulation with ADP, platelets were pre-incubated with 1, 10, 100 or 1000nM of the $P2Y_1$ receptor antagonists MRS2179 or MRS2500. MRS2179 demonstrated a dose-dependent attenuation of platelet induced PMN cell migration towards MDC compared to vehicle controls (Veh: 3.65 ± 0.26 vs. 1nM: 3.26 ± 0.24, 10nM: 2.35 ± 0.36 $P<0.01$, 100nM: 2.00 ± 0.26 $P<0.001$, 1000nM: 1.46 ± 0.27 $P<0.001$, **Figure 4.20A**). This dose dependent attenuation of PMN cell migration was replicated with MRS2500 treatment (Veh:2.21 ± 0.12 vs. 1nM: 2.50 ± 0.20, 10nM: 2.45 ± 0.28, 100nM: 2.34 ± 0.35, 1000nM: 1.58 ± 0.15 $P<0.01$, **Figure 4.20B**). Furthermore, supporting the observations of the previous *in vivo* models, pre-treatment with the $P2Y_{12}$ receptor antagonist MRS2395 failed to affect platelet induced PMN chemotaxis at any measured dose (Veh: 2.35 ± 0.22, 1nM: 1.66 ± 0.26, 10nM: 1.86 ± 0.21, 100nM: 1.82 ± 0.18, 1000nM: 1.80 ± 0.32, **Figure 4.20C**), a lack of efficacy on PMN chemotaxis also replicated with AR-C66096 (Veh: 2.21 ± 0.12 vs. 1nM: 1.81 ± 0.11, 10nM: 2.63 ± 0.31, 100nM: 2.31 ± 0.29, 1000nM: 2.08 ± 0.11, **Figure 4.20D**).

These results further support the observations concluded from the model of allergic inflammation *in vivo* that the $P2Y_1$ but not the $P2Y_{12}$ receptor is critical in platelet induced PMN migration both *in vitro* and *in vivo.*

Significant platelet-induced leukocyte migration was also observed towards the chemokines f-MLP (Veh: 1.00 ± 0.46 vs. 100nM f-MLP: 3.00 ± 0.53, $P < 0.001$, **figure 4.21A**) and SDF-1 (Veh: 1.00 ± 0.11 vs. 100nM SDF-1: 2.01 ± 0.17, $P < 0.001$, **figure 4.21B**). In agreement with data observed for platelet-induced leukocyte migration towards MDC, inhibition of the $P2Y_1$ receptor with the antagonists MRS2179 and MRS2500 significantly inhibited platelet-induced PMN migration towards f-MLP compared to vehicle controls (Veh: 3.00 ± 0.53 vs.1000nM MRS2179: 1.10 ± 0.10, $P < 0.001$; 1000nM MRS2500: 1.16 ± 0.18, $P < 0.001$) whilst no effect was observed with either of the $P2Y_{12}$ receptor antagonists (Veh: 3.00 ± 0.53 vs. 1000nM MRS2395: 2.48 ± 0.20; AR-C66096: 3.06 ± 0.11, **figure 4.21A**).

Surprisingly however, neither $P2Y_1$ nor $P2Y_{12}$ receptor antagonism was capable of inhibiting platelet induced PMN migration towards SDF-1α (Veh: 2.01 ± 0.17 vs. 1000nM MRS2179: 1.91 ± 0.15; 1000nM MRS2500: 2.09 ± 0.19; 1000nM MRS2395: 1.80 ± 0.16; 1000nM AR-C66096: 2.52 ± 0.17, **figure 4.21B**).

Finally, pre-incubation of platelets with the $P2X_1$ receptor antagonist NF-279 failed to modulate PMN cell chemotaxis towards MDC in the presence of platelets at any measured concentration compared to the positive controls (Veh: 3.46 ± 0.23 vs. 1nM: 3.02 ± 0.26, 10nM: 3.34 ± 0.26, 100nM: 2.90 ± 0.24, 1000nM: 3.14 ± 0.14) supporting the earlier *in vivo* observations (**figure 4.22**).

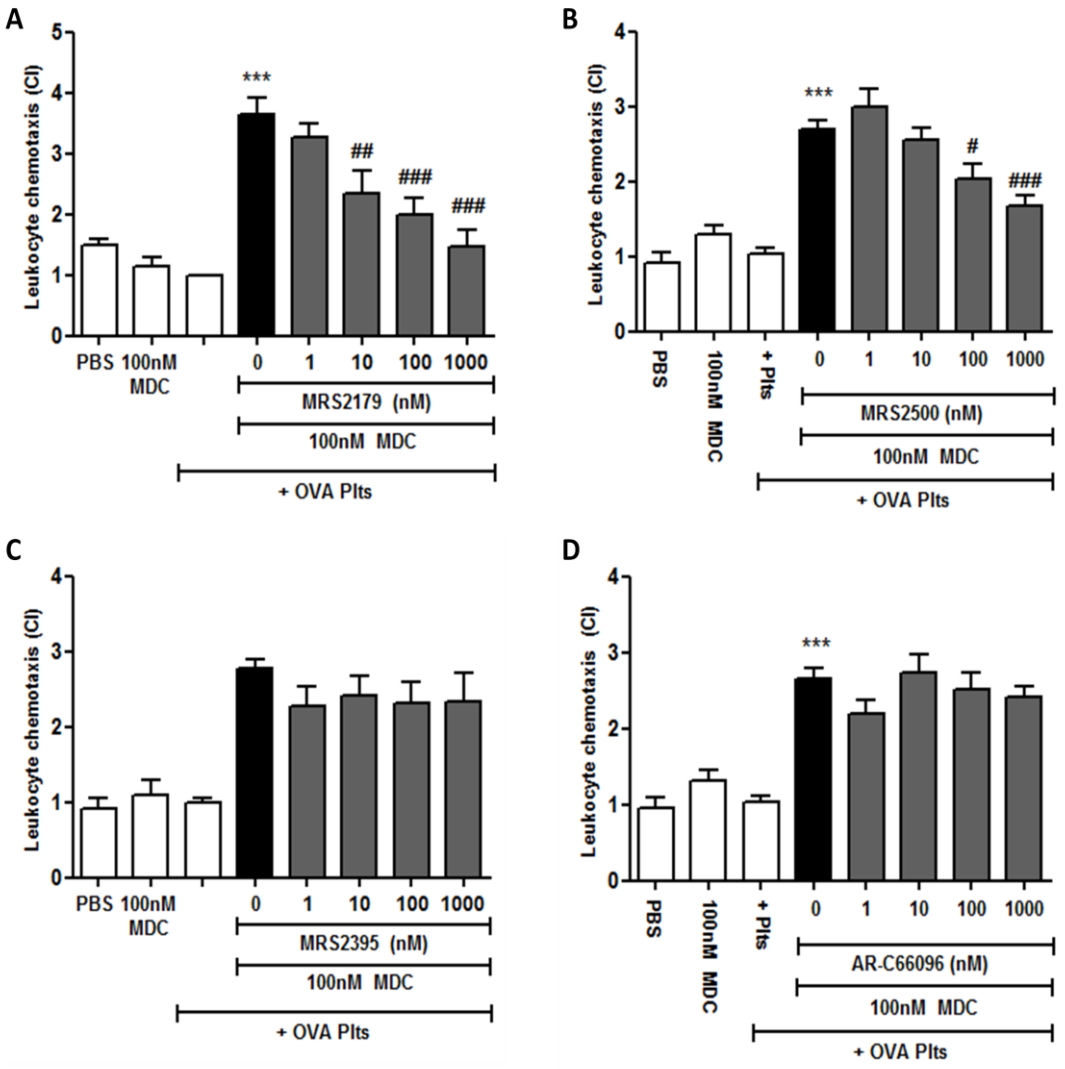


Figure 4. 20 The Effects of $P2Y_1$ and $P2Y_{12}$ Receptor Antagonism on Platelet Induced PMN Chemotaxis *in vitro*

Platelets and PMNs were isolated from citrated blood and bone marrow from 1.5mg/kg OVA sensitised mice. Platelets were then pre-incubated with 1, 10, 100 and 1000 of the $P2Y_1$ receptor antagonists MRS2179 (**A**) and MRS2500 (**B**), or the $P2Y_{12}$ receptor antagonists MRS2365 (**C**) and AR-C66096 (**D**) before subsequent stimulation with 100nM ADP before mixing with PMN cells. The cell mixture was added to the top well of a chemotaxis chamber. PMN chemotaxis towards MDC (bottom well 100nM) was then quantified after a 90 minute incubation and analysed as a chemotactic index. Data are expressed as mean ± SEM. *** = $P < 0.001$ towards sham controls, # = $P < 0.05$, ## = $P < 0.01$, ### = $P < 0.001$ towards positive controls. n = 6-9.

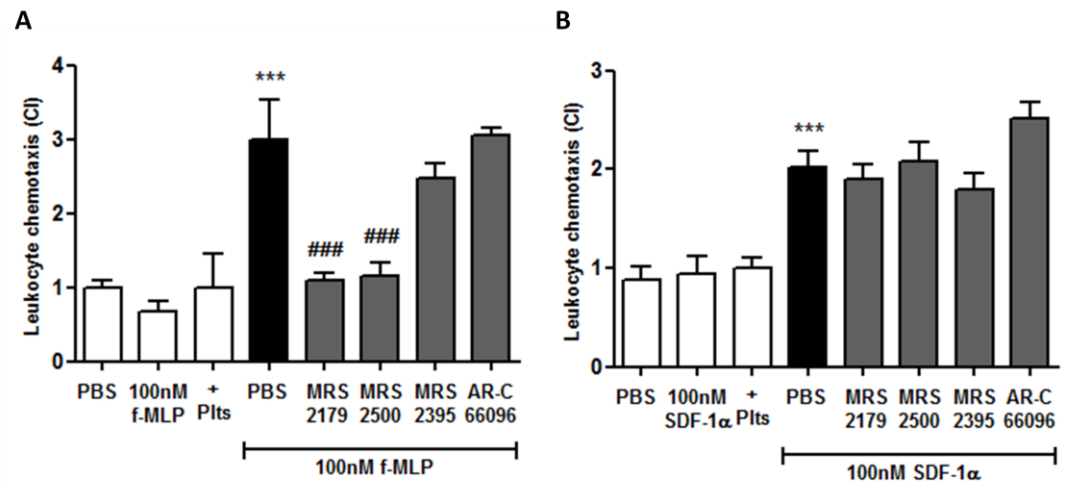


Figure 4. 21 The Effects of $P2Y_1$ and $P2Y_{12}$ Receptor Antagonism on Platelet Induced PMN Chemotaxis *in vitro*

Platelets and PMNs were isolated from citrated blood and bone marrow from 1.5mg/kg OVA sensitised mice. Platelets were then pre-incubated with 1000nM of either the $P2Y_1$ receptor antagonists MRS2179, MRS500 or the $P2Y_{12}$ receptor antagonists MRS2395 or AR-C66096 before subsequent stimulation with 100nM ADP and mixing with PMN cells. The cell mixture was added to the top well of a chemotaxis chamber. PMN chemotaxis was measured towards either 100nM f-MLP (**A**) or 100nM SDF-1 (**B**) after a 90 minute incubation and analysed as a chemotactic index. Data are expressed as mean ± SEM. *** = $P < 0.001$towards sham controls, ### = $P < 0.001$ towards positive controls n = 4-8.

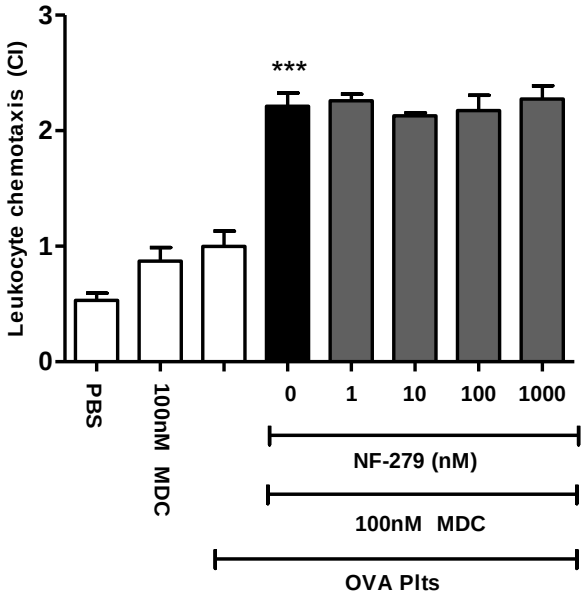


Figure 4. 22 The Effects of $P2X_1$ Receptor Antagonism on Platelet Induced PMN Chemotaxis *in vitro*

Platelets and PMNs were isolated from citrated blood and bone marrow from 1.5mg/kg OVA sensitised mice. Platelets were then pre-incubated with 1, 10, 100 and 1000 NF-279 before subsequent stimulation with 100nM ADP before mixing with PMN cells. The cell mixture was added to the top well of a chemotaxis chamber. PMN chemotaxis towards MDC (bottom well 100nM) was then quantified after a 90 minute incubation and analysed as a chemotactic index. Data are expressed as mean ± SEM. *** = $P < 0.001$ where indicated to column 3,n = 6-9.

4.9. An Investigation into the Role of RhoA in Inflammatory Processes Downstream of Purinergic Receptors

In the previously described experiments, the role of platelet $P2Y_1$ receptor signalling has been shown to be critical in the inflammatory actions of platelets, whilst the $P2Y_{12}$ receptor signalling has been shown to only be implicated in thrombotic process of platelets. It was therefore important to try to identify differences in the downstream signalling of the purinergic receptors that separates this role from that of platelet aggregation.

RhoA is a small GTPase that belongs to the Rho family of GTPases, a family of GTP binding proteins critically involved in the regulation of the platelet cytoskeleton and platelet function (Sakumura *et al* 2005). In its active form RhoA binds to and activates Rho-associated coiled-coil forming kinases (ROCKs) (Ridley, 2013b). Once activated, ROCK phosphorylates and inactivates myosin light chain (MLC) phosphatase resulting in an overall increase in MLC phosphorylation initiating platelet shape change (Zhu *et al* 2011). Indeed RhoA has been shown to be involved in platelet shape change, filopodia formation and granule secretion (Aslan *et al* 2013, Bustelo *et al* 2007, Pleines *et al* 2012). Similar processes also appear to be necessary in leukocytes for cell adhesion whilst in conjunction with other GTPases of the Rho family (Rac1, cdc42) coordinates cell movement (Aslan *et al* 2013, Sakumura *et al* 2005). However, RhoA appears to be redundant in platelet aggregation (Eckly et al., 2001; Pleines et al., 2012). Therefore, given that $P2Y_1$ activation of platelets in the context of allergic inflammation does not increase aggregation per sé, the significance of this redundant pathway in the context of the allergic response to allergen was investigated.

In addition to the described role of RhoA in platelet function, activation of Rho kinase isoforms has been demonstrated in lung endothelial cells during inflammation (Mong *et al* 2009), whilst inhibition of Rho-Kinase through the use of pharmacological antagonists inhibits airway hyperresponsiveness, inflammation, extra-cellular matrix remodelling and decrease neutrophil accumulation to the lungs in models of abdominal sepsis and chronic inflammation (Palani *et al* 2012, Souza Possa *et al* 2012). Given these previously described roles of RhoA signalling for inflammatory cell recruitment; the possibility of a role for RhoA in platelet function during inflammatory events was investigated.

4.9.1 An Investigation into the Impact of RhoA Phosphorylation at S188 Following Allergen Challenge

RhoA phosphorylation was used as a biomarker of activity within the RhoA system. Female Balb/c mice were sensitised and challenged to OVA as described in figure 2.2. Animals were treated with either vehicle, 3mg/kg MRS2500 or AR-C66096. 6 hours post final allergen challenge, citrated blood was collected via cardiac puncture. Platelets were isolated and phosphorylated RhoA was measured through western blot using the anti-pRhoA S188 antibody. Allergen sensitisation and challenge resulted in RhoA phosphorylation at S188, a process that was not observed in sham sensitised controls. Interestingly, RhoA S188 phosphorylation was specifically suppressed through pre-treatment with MRS2500 but not AR-C66096, providing a possible basis for modulation of RhoA signalling in the $P2Y_1$ specific role in the inflammatory actions of platelets (**Figure 4.23)**.

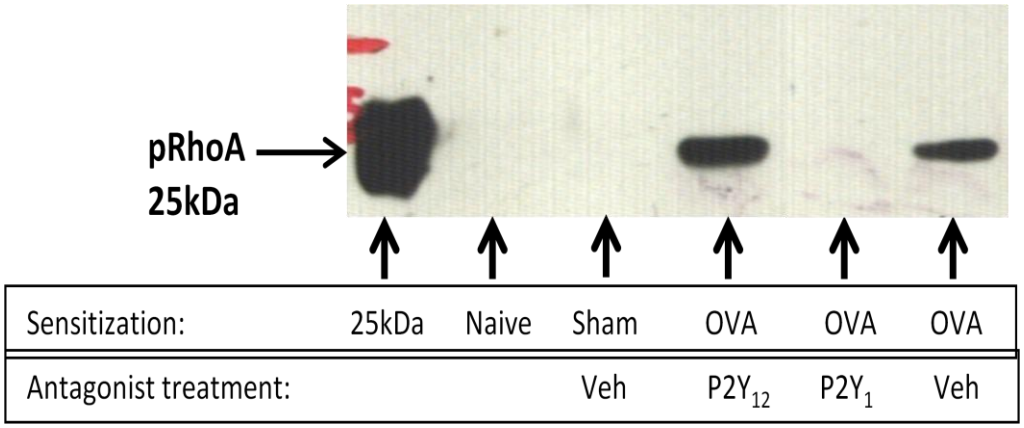


Figure 4. 23 Effects of $P2Y_1$ and $P2Y_{12}$ Antagonism on RhoA Phosphorylation at S188

Female Balb/c mice were sensitised and challenged to OVA as described in figure 2.2. Animals were treated with either vehicle or 3mg/kg MRS2500 or AR-C66096. 6 hours post final allergen challenge, citrated blood was collected via cardiac puncture. Platelets were isolated and phosphorylated RhoA was measured through western blot using the anti-pRhoA S188 antibody.

4.9.2 An Investigation into the Role of Platelet RhoA Signalling in Pulmonary Leukocyte Recruitment *in vivo*

Pre-treatment of some groups of mice with 20mg/kg Busulfan induced significant thrombocytopenia (Veh:1.68 ± 0.06x10^9 plts/ml vs. 20mg/kg Busulfan: 0.50 ± 0.02x10^9 plts/ml, P < 0.001, **figure 4.24**).

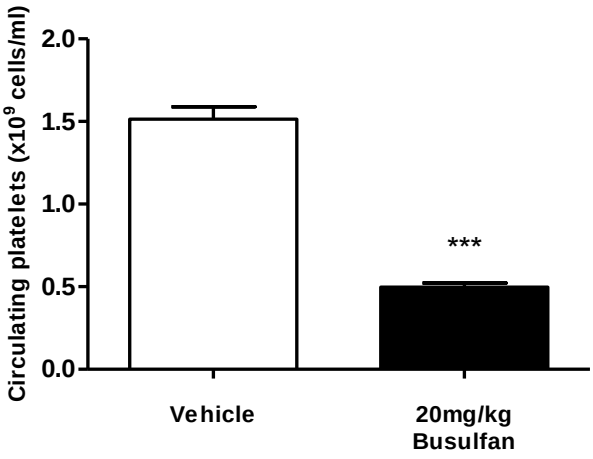


Figure 4. 24 Busulfan Induced Thrombocytopenia

Female Balb/c mice were treated with either vehicle or 20mg/kg Busulfan. 10 days post final dosing, circulating platelet counts were quantified under 40X magnification on a light microscope. Data expressed as mean ± SEM, N = 5, *** = P < 0.001.

Busulfan induced thrombocytopenia significantly attenuated allergen-induced total leukocyte recruitment which was restored following reinfusion of 2.36x10^8 washed platelets per mouse (OVA: 406.50 ± 51.33x10^4 cells/ml vs. OVA + Busulfan: 134.50 ± 24.19x10^4 cells/ml, P < 0.001; WP: 309.00 ± 23.87x10^4 cells/ml, **figure 4.25A**). This attenuation of leukocyte recruitment in thrombocytopenic mice and subsequent restoration with WP was restored in macrophage counts (OVA: 91.01 ± 16.58x10^4 cells/ml vs. OVA + Busulfan: 54.13 ± 16.05x10^4 cells/ml; WP: 50.04 ± 5.56x10^4 cells/ml, **figure 4.25B**), infusion with WP restored lymphocyte counts (OVA: 9.65 ±

$3.48x10^4$ cells/ml vs. OVA + Busulfan: $3.68 \pm 1.49x10^4$ cells/ml; WP: 5.70 ± 1.43, **figure 4.25C**), neutrophil counts (OVA: $47.52 \pm 13.65x10^4$ cells/ml vs. OVA + Busulfan: $8.35 \pm 2.82x10^4$ cells/ml, $P < 0.05$; WP: $50.20 \pm 9.63x10^4$ cells/ml, **figure 4.25D**) and eosinophil counts (OVA: $258.52 \pm 25.03x10^4$ cells/ml vs. OVA + Busulfan: $38.49 \pm 19.28x10^4$ cells/ml, $P < 0.001$; WP: $196.06 \pm 17.63x10^4$ cells/ml, **figure 4.25E**).

Reinfusion with platelets pre-treated with 100μM of the Rho kinase inhibitor GSK429286 reinstated suppression of total leukocyte recruitment (OVA: $406.50 \pm 51.33x10^4$ cells/ml vs. GSK429286: $174.50 \pm 32.23x10^4$ cells/ml, $P < 0.001$ **figure 4.25A**), a process which was not replicated in macrophages (OVA: $91.01 \pm 16.58x10^4$ cells/ml vs. GSK429286: $86.64 \pm 16.18x10^4$ cells/ml, **figure 4.25B**), but in lymphocytes (OVA: $9.65 \pm 3.48x10^4$ cells/ml vs. GSK429286: $0.20 \pm 0.20x10^4$ cells/ml, $P < 0.01$ **figure 4.25C**), neutrophils (OVA: $47.32 \pm 13.65x10^4$ cells/ml vs. GSK429286: $25.47 \pm 7.34x10^4$ cells/ml, **figure 4.26D**) and eosinophil numbers (OVA: $258.22 \pm 25.03x10^4$ cells/ml vs. GSK429286: $62.01 \pm 11.91x10^4$ cells/ml, $P < 0.001$ **figure 4.25E**). This demonstrates a significant role of platelet RhoA signalling in pulmonary leukocyte recruitment in this murine model of allergic inflammation.

Given the similarity of the results obtained through inhibition of RhoA with GSK429286 and the inhibition of $P2Y_1$ receptors with MRS2179 and MRS2500, further studies investigated if RhoA signalling was downstream of the $P2Y_1$ receptor in platelet signalling.

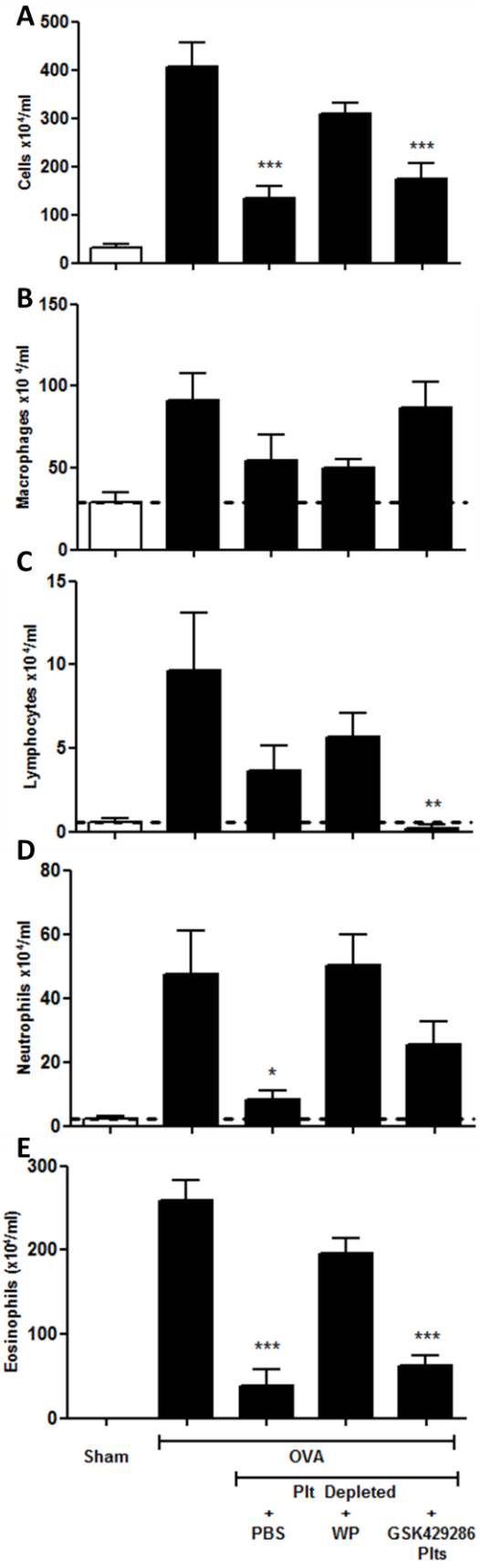


Figure 4. 25 The Effects of GSK429286 Treated Platelets on Pulmonary Leukocyte Recruitment

Mice were rendered thrombocytopenic through a dosing protocol involving the bone marrow depleting agent Busulfan. During this process mice were subjected to a two week sensitisation (1.5mg/kg OVA) and allergen exposure protocol. Washed platelets isolated from allergen sensitised mice were pre-treated with either vehicle or 100µM GSK429286. Treated platelets were subsequently reinfused in thrombocytopenic mice prior to allergen exposure on days 14 and 15. Lavage fluid was collected 24 hours post final allergen exposure (day 17). **A** Total BAL Leukocytes **B** Macrophages **C** Lymphocytes **D** Neutrophils **E** Eosinophils. Data: Mean ± SEM, N= 5 animals per group. * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$.

4.9.3 An Investigation into the Role of RhoA Signalling Downstream of the $P2Y_1$ Receptor in Platelet Induced PMN Cell Migration

The ability of RhoA signalling downstream of platelet $P2Y_1$ receptor activation to induce the activation and motility of murine bone marrow PMN cells to MDC was investigated. Stimulation of platelets with 100nM ADP or 1000nM of the $P2Y_1$ receptor agonist MRS2365 induced significant PMN cell migration towards 100nM MDC (Vehicle: 1.32 ± 0.26 vs. 100nM ADP: 3.16 ± 0.14, $P<0.001$; 1000nM MRS2365: 2.52 ± 0.43, $P<0.01$, **Figure 4.26**). ADP induced increases in platelet-induced PMN cell migration were attenuated by treatment with the RhoA antagonist GSK429286 (100µM GSK: 1.52 ± 0.43, $P<0.01$, **Figure 4.26**). This was replicated in groups stimulated with the specific $P2Y_1$ agonist (MRS2365) with subsequent treatment with GSK429286 (100µM GSK: 1.32 ± 0.49, $P<0.05$, **Figure 4.26**).

This collection of data confirms that platelet induced PMN migration towards MDC is a ADP-dependent process, furthermore the suppression of either ADP or MRS2365 induced PMN cell migration by GSK429286 suggests that RhoA signalling downstream of the $P2Y_1$ receptor is necessary.

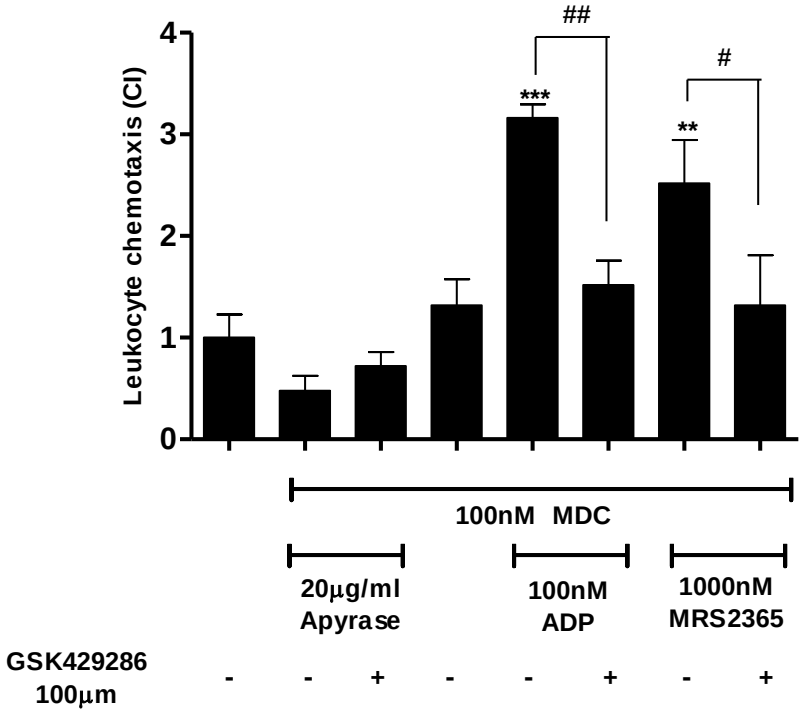


Figure 4. 26 RhoA Signalling Downstream of $P2Y_1$ Stimulation is Necessary in Platelet Induced PMN Cell Migration

Platelets and PMNs were isolated from citrated blood and bone marrow from 1.5mg/kg OVA sensitised mice. Platelets were then pre-incubated either vehicle, 20µg/ml apyrase or 100µM GSK429286 before subsequent stimulation with either 100nM ADP or 1000nM MRS2365 before mixing with PMN cells. The cell mixture was added to the top well of a chemotaxis chamber. PMN chemotaxis towards MDC (bottom well 100nM) was then quantified after a 90 minute incubation and analysed as a chemotactic index. Data are expressed as mean ± SEM. * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.01$ where indicated to column 1, # = $P < 0.05$, ## = $P < 0.01$ n 4.

Given that GSK429286 is a ROCK inhibitor. To further investigate the impact of RhoA signalling downstream of the $P2Y_1$ receptor a second known direct inhibitor of RhoA signalling was used. Botulinum ADP-ribosyltransferase (C3 exoenzyme), is known to directly inhibit RhoA activity in human platelets (Morii et al., 1992), this C3 exoenzyme was therefore used to determine if RhoA specific activity was involved in platelet induced leukocyte chemotaxis *in vitro* to ADP or specific activation of the $P2Y_1$ receptor using the agonist MRS2365 (**Figure 4.27**).

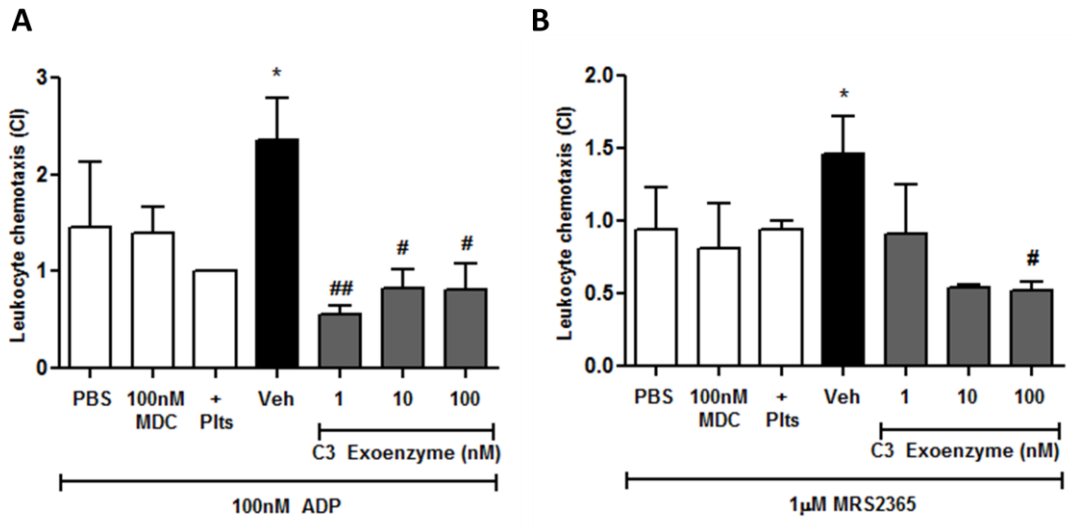


Figure 4. 27. The effects of C3 exoenzyme on platelet induced leukocyte chemotaxis *in vitro*

Platelets and PMNs were isolated from citrated blood and bone marrow from Balb/c mice. Platelets were then pre-incubated either vehicle, 1, 10 or 100nM C3 Exoenzyme before subsequent stimulation with either 100nM ADP (**A**) or 1000nM MRS2365 (**B**) before mixing with PMN cells. The cell mixture was added to the top well of a chemotaxis chamber. PMN chemotaxis towards MDC (bottom well 100nM) was then quantified after a 90 minute incubation and analysed as a chemotactic index. Data are expressed as mean ± SEM. * = $P < 0.05$, ## = $P < 0.01$, ### = $P < 0.01$ where indicated to column 1, n 4.

Initial chemotaxis assays were performed on platelets and PMNs isolated from blood and bone marrow of female balb/c mice. Platelets were incubated with the RhoA inhibitor C3 exoenzyme prior to stimulation with either 100nM ADP (**figure 4.27A**) or 1μM of the $P2Y_1$ specific agonist MRS2365 (**figure 4.27)**. Post stimulation with ADP or MRS2365, platelets were added to PMN cells to induce migration towards 100nM MDC. No significant migration was observed to leukocytes alone following either ADP stimulation (1.40 ± 0.68) or MRS2365 stimulation (0.94 ± 0.30), leukocyte migration towards MDC alone following either ADP stimulation (1.40 ± 0.26) or MRS2365 stimulation (0.81 ± 0.31) or leukocytes in the presence of

platelets following ADP stimulation (1.00 ± 0.00) or MRS2365 stimulation (1.00 ± 0.00).

When platelets were mixed with PMNs, significant migration to 100nM MDC was observed following stimulation by both ADP (2.36 ± 0.43, $P < 0.05$, **figure 4.27A**) and MRS2365 stimulation (1.57 ±0.22, $P < 0.05$, **figure 4.27B**). Pre-incubation of platelets with C3 exoenzyme significantly attenuated platelet induced leukocyte migration towards MDC following ADP stimulation (Veh: 2.36 ± 0.43 vs.1nM: 0.56 ± 0.10, P<0.01; 10nM: 0.84 ± 0.19, P<.0.05; 100nM: 0.82 ± 0.27, P<0.05, **figure 4.27A**). Importantly, this inhibition of platelet induced PMN migration by C3 exoenzyme was also observed downstream of specific $P2Y_1$ receptor activation using the $P2Y_1$ specific receptor agonist MRS2365 (Veh: 1.56 ± 0.22 vs. 1nM: 0.92 ± 0.34; 10nM: 0.54 ± 0.02; 100nM:0.53 ± 0.06, P<0.05, **figure 4.27B**). In agreement with inhibition of platelet induced PMN migration with pre-treatment with the ROCK inhibitor GSK429286, this demonstrates that RhoA signalling downstream of the $P2Y_1$ receptor is critical in platelet induced PMN migration *in vitro.*

Chapter V

Results III

Investigating the Dichotomy in Platelet Function

5.1 An investigation into the Regulation of the Apparent Dichotomy of Platelet Function in Hemostasis and Inflammation

As has been previously described, platelets are capable of being activated by a number of different 'platelet agonists', for example the classical agonists of ADP, Thrombin and TxA_2 and adrenaline. However platelets also express a number of receptors for inflammatory mediators, including SDF-1α, MDC, IL-1β and LPS. Indeed, Clemetson *et al* 2000 have demonstrated functional expression of the SDF-1α receptor CXCR4 and the MDC receptor CCR4 on the surface of platelets (Clemetson et al., 2000) and the LPS receptors TLR2 and TLR4 (Semple and Freedman, 2010), whilst more recent studies have identified the IL-1β specific receptor IL-1R1 on the platelets surface (Brown *et al*. 2013).

Given the dichotomy in platelet activation leading to pro-inflammatory events compared to aggregation described in Chapter IV, and in numerous inflammatory conditions such as asthma, COPD, atherosclerosis, rheumatoid athritis and ezcema, platelet activation leads to the formation of circulating platelet-leukocyte aggregates via adhesion molecule interactions which do not lead to platelet aggregation per sé (Ott I, Neumann FJ, Gawaz M, Schmitt M, 1996; Neumann et al., 1997; Joseph et al., 2001; Sarma, 2002; Huo et al., 2003; Bunescu et al., 2004; Pitchford et al., 2004, 2005; Irving et al., 2008), this body of evidence suggests that platelet activation as a result of inflammatory stimuli may result in different parameters of platelet function.

In this chapter, using platelets isolated from human venous blood a series of *in vitro* assays of platelet function covering aspects of inflammation and hemostasis (platelet migration, platelet-leukocyte conjugate formation, platelet P-selectin expression and platelet aggregation) are used to investigate the impact of both inflammatory and haemostatic stimuli (SDF-1α, MDC, f-MLP, 5-HT, LPS, IL-1β, Adrenaline, the

TxA_2 analogue U46619, TRAP and ADP) on platelet function to elucidate the differential signalling mechanisms involved.

5.2 An Investigation into the Effects of the Stimulation of Platelets by Inflammatory Mediators on Platelet Function

SDF-1α (CXCL12) is a chemokine known to activate platelet expressed CXCR4 receptors. Activation of the CXCR4 receptor activates a complex cascade of intracellular signalling pathways regulating and promoting cell migration, adhesion, survival and proliferation. Indeed, platelet migration has previously been observed towards SDF-1α in a CXCR4 dependent fashion, as well as inhibiting cAMP production (Kowalska et al., 2000; Gear, 2001; Kraemer et al., 2010). Using the aforementioned assays of platelet function (both haemostatic and inflammatory), the role of SDF-1 on platelet function was investigated.

Stimulation with SDF-1α failed to induce any change in platelet P-selectin expression compared to sham controls in either percent expression (Veh: 6.49 ± 2.08% vs. 0.1µg/ml SDF-1α: 7.25 ± 3.22%; 1µg/ml SDF-1α: 8.72 ± 2.40% and 10µg/ml SDF-1α: 7.04 ± 0.01, **figure 5.1A**) or MFI on P-selectin expression (Veh: 0.47 ± 0.09 vs. 0.1µg/ml SDF-1α: 0.39 ± 0.01; 1µg/ml SDF-1α: 0.50 ± 0.10 and 10µg/ml SDF-1α: 0.46 ± 0.00, **figure 5.1B**).

Furthermore, SDF-1α failed to significantly enhance the formation of platelet leukocyte conjugates in human venous whole blood when compared to sham controls, either in a fold increase in the incidence of conjugates (Veh: 1.04 ± 0.04 vs. 0.1µg/ml SDF-1α: 1.26 ± 0.15; 1µg/ml SDF-1α: 1.19 ± 0.13 and 10µg/ml SDF-1α: 1.53 ± 0.22, **figure 5.1C**) or MFI on conjugate formation (Veh: 3.63 ± 0.62 vs.

0.1µg/ml SDF-1α: 3.15 ± 0.57; 1µg/ml SDF-1α: 3.16 ± 0.58 and 10µg/ml SDF-1α: 3.18 ± 0.61, **figure 5.1D**).

SDF-1α stimulation was also not capable of inducing platelet aggregation in gel filtered isolated platelets *in vitro* (Veh: 0.77 ± 0.53% vs. 0.1µg/ml SDF-1α: 1.30 ± 0.90; 1µg/ml SDF-1α: 3.15 ± 1.76% and 10µg/ml SDF-1α: 3.67 ± 1.90%, **figure 5.1E**).

In contrast, whilst SDF-1α stimulation had no effect on P-selectin expression, the formation of platelet-leukocyte aggregates or platelet aggregation, SDF-1α significantly elevated platelet migration compared to vehicle control and platelets fixed with 4% PFA prior to stimulation to provide a suitable control for chemokine stimulation (Veh: 1.00 ± 0.00 vs. 0.1µg/ml SDF-1α: 1.37 ± 0.26; 0.5µg/ml SDF-1α: 1.48 ± 0.23; 1µg/ml SDF-1α: 2.49 ± 0.24, $P < 0.001$ and PFA treated 1µg/ml SDF-1α: 1.11 ± 0.07, **figure 5.1F**).

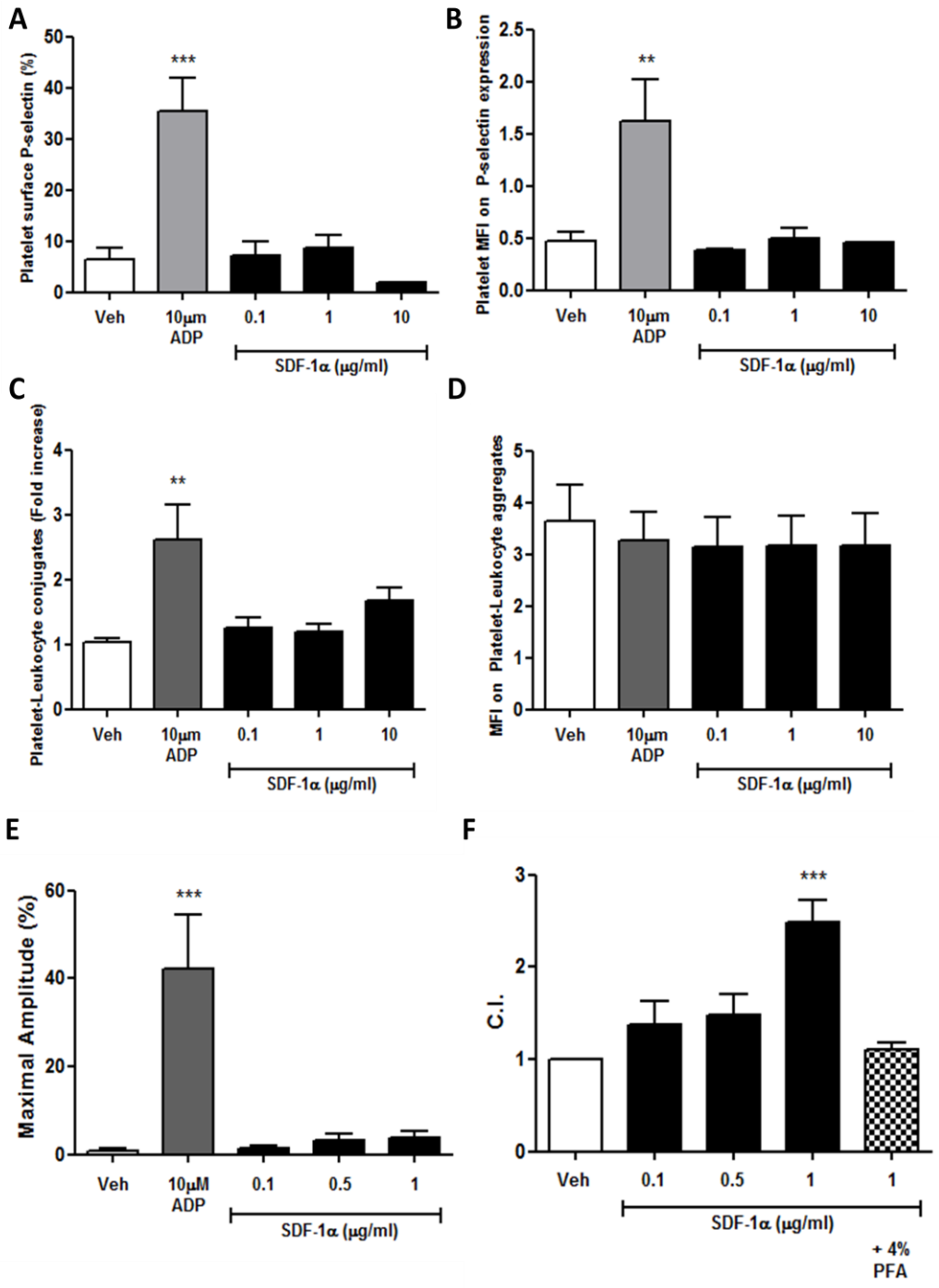


Figure 5. 1. Investigating the effects of the CXCR4 chemokine SDF-1α on parametres of platelet activation

Stimulation with 0.1, 1 or 10µg/ml SDF-1 on gel filtered platelets isolated from human venous blood exposed to showed increased P-selectin activity (**A:** %, **B**: MFI), on platelet leukocyte conjugation in human venous whole blood (**C**: %, **D**: MFI), platelet aggregation in gel filtered platelets (**E**) and platelet migration with washed platelets isolated from human venous blood (**F**) Data: Mean ± SEM, N = 5. ***** = P < 0.05, ****** = P < 0.01**, ***** = P < 0.001 compared to vehicle controls.

MDC stimulates platelet CCR4 receptors, and has been shown to induce intracellular Ca^{2+} mobilisation and in some cases an elevation in P-selectin expression (Kowalska et al., 2000; Gear, 2001). Using the aforementioned assays of platelet function, the role of MDC on both the inflammatory and haemostatic parameters of platelet activation was investigated.

Similar to the effects seen by SDF-1α stimulation, MDC stimulation was unable to induce elevations in platelet surface P-selectin expression compared to vehicle controls in either percent expression (Veh 4.01 ± 0.33% vs. 0.1µg/ml MDC: 4.36 ± 0.46%; 1µg/ml MDC: 5.55 ± 1.14% and 10µg/ml MDC: 5.98 ± 1.27%, **figure 5.2A**) or MFI on P-selectin expression (Veh: 0.48± 0.08 vs. 0.1µg/ml MDC: 0.50 ± 0.06; 1µg/ml: 0.56 ± 0.10 and 10µg/ml MDC: 0.57 ± 0.10, **figure 5.2B**).

MDC also failed to induce a significant increase in the formation of platelet-leukocyte conjugates in human venous whole blood, however a trend depicting a small increase in conjugate formation is visible in the total circulating numbers (Veh: 1.05 ± 0.04 vs. 0.1µg/ml MDC: 1.02 ± 0.07; 1µg/ml MDC: 1.04 ± 0.03 and 10µg/ml 1.30 ± 0.19, **figure 5.2C**) but not the MFI on platelet leukocyte conjugates (Veh: 4.09 ± 1.83 vs. 0.1µg/ml MDC: 3.64 ± 1.63; 1µg/ml MDC: 3.62 ± 1.62 and 10µg/ml MDC: 3.64 ± 1.63, **figure 5.2D**).

Stimulation of gel filtered isolated platelets failed to induce any significant platelet aggregation, however once again, a small trend appeared to show a small increase in MDC induced platelet aggregation compared to negative controls (Veh: 11.52 ± 5.06% vs. 0.1µg/ml MDC: 11.67 ± 9.17; 1µg/ml MDC: 15.67 ± 2.88 and 10µg/ml MDC: 23.50 ± 2.85, **figure 5.2E**).

Finally, stimulation of washed platelets isolated from citrated blood induced significant platelet migration compared to vehicle and fixed platelet controls (Veh:1.00 ± 0.06 vs. 0.1µg/ml MDC: 0.94 ± 0.13; 1µg/ml MDC: 1.83 ± 0.13, $P < 0.01$; 10µg/ml MDC: 1.39 ± 0.00 and PFA treated 10µg/ml MDC: 0.58 ± 0.00, **figure 5.2F**).

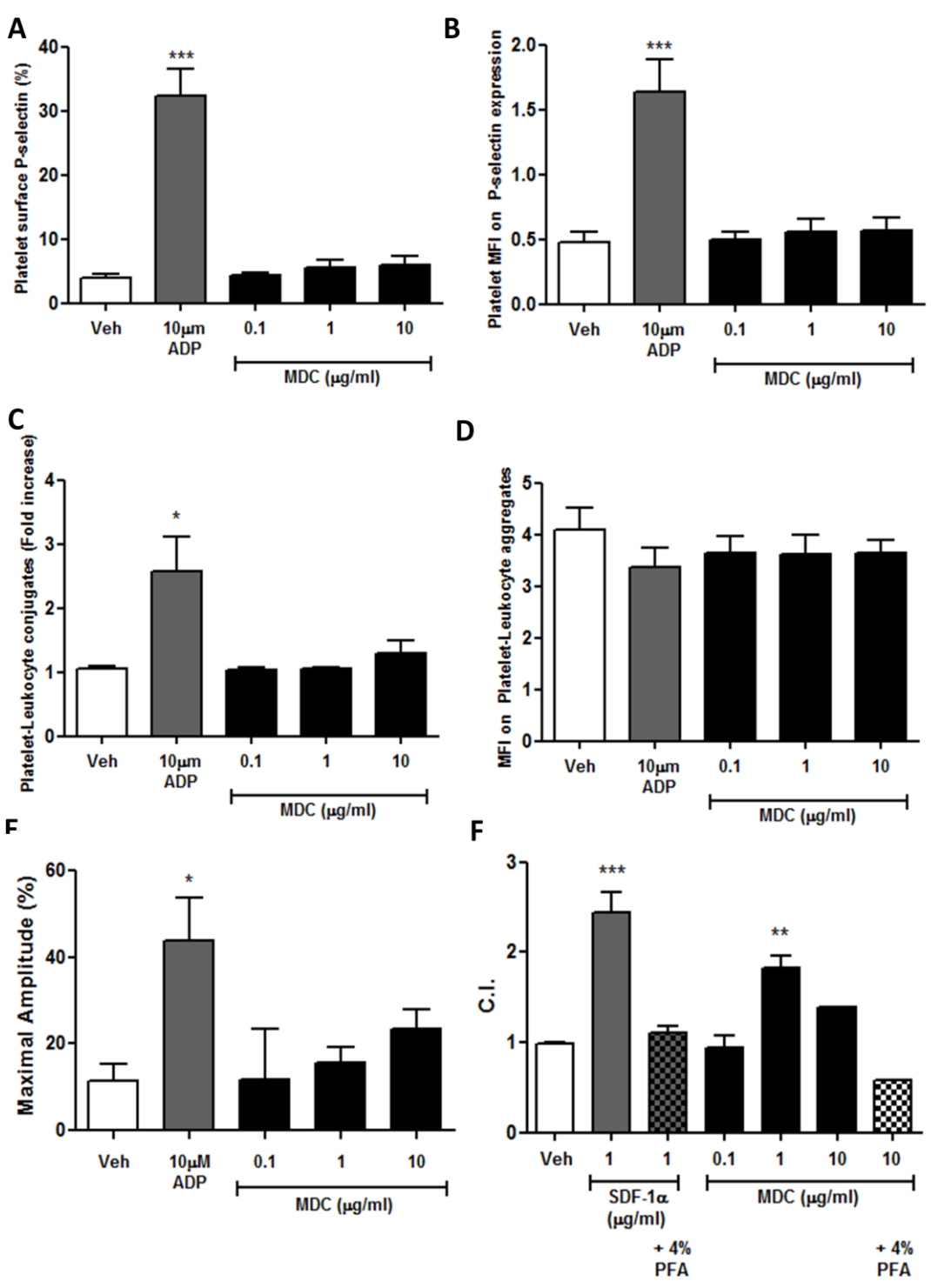


Figure 5. 2 Investigating the effects of the CCR4 chemokine MDC on parameters of platelet activation

Stimulation with 0.1, 1 or 10µg/ml MDC on gel filtered platelets isolated from human venous blood exposed to showed increased P-selectin activity (**A:** %, **B**: MFI), on platelet leukocyte conjugation in human venous whole blood (**C**: %, **D**: MFI), platelet aggregation in gel filtered platelets (**E**) and platelet migration with washed platelets isolated from human venous blood (**F**) Data: Mean ± SEM, N = 5. ***** = $P < 0.05$, ****** = $P < 0.01$**,** ******* = $P < 0.001$ compared to vehicle controls.

f-MLP stimulates functional formyl peptide receptors (FPRs) on human platelets, and has previously been implicated in platelet chemotaxis (Czapiga et al., 2005). Using the aforementioned assays of platelet function, the role of MDC on both the inflammatory and haemostatic parameters of platelet activation was investigated.

f-MLP stimulation was unable to induce elevations in platelet surface P-selectin expression compared to vehicle controls in either percent expression (Veh 4.73 ± 0.90% vs. 0.1µM f-MLP: 5.57 ± 1.37%; 1µM f-MLP: 4.54 ± 0.69% and 10µM f-MLP: 4.26 ± 0.67%, **figure 5.3A**) or MFI on P-selectin expression (Veh: 0.50 ± 0.08 vs. 0.1µM f-MLP: 0.52 ± 0.08; 1µM: 0.49 ± 0.08 and 10µM: 0.43 ± 0.04, **figure 5.3B**). Furthermore, f-MLP stimulation failed to induce an increase in the formation of platelet-leukocyte conjugates in human venous whole blood, both in terms of a fold increase compared to vehicle control (Veh: 1.05 ± 0.04 vs. 0.1µM f-MLP: 1.34 ± 0.19; 1µM f-MLP: 1.44± 0.22 and 10µM f-MLP: 1.41 ± 0.14, **figure 5.3C**) and MFI on platelet leukocyte conjugates (Veh:4.14 ± 0.30 vs. 0.1µM f-MLP: 4.18 ± 0.45; 1µM f-MLP: 4.50 ± 0.32 and 10µM f-MLP: 4.62 ± 0.43, **figure 5.3D**).

Stimulation of gel filtered isolated platelets also failed to induce any visible changes in platelet aggregation, compared to negative controls (Veh: 0.00 ± 0.00% vs. 0.1µM f-MLP: 0.00 ± 0.00; 1µM f-MLP: 0.00 ± 0.00 and 10µM f-MLP: 0.00 ± 0.00, **figure 5.3E**).

Finally, washed platelets isolated from citrated blood demonstrated significant platelet migration towards f-MLP compared to vehicle and fixed platelet controls (Veh: 1.00 ± 0.00 vs. 0.1µM f-MLP: 1.72 ± 0.22, $P < 0.05$; 1µM f-MLP: 1.68 ± 0.22, $P < 0.05$; 10µM f-MLP: 1.45 ± 0.25 and PFA treated 10µ f-MLP: 1.14 ± 0.00, **figure 5.3F**).

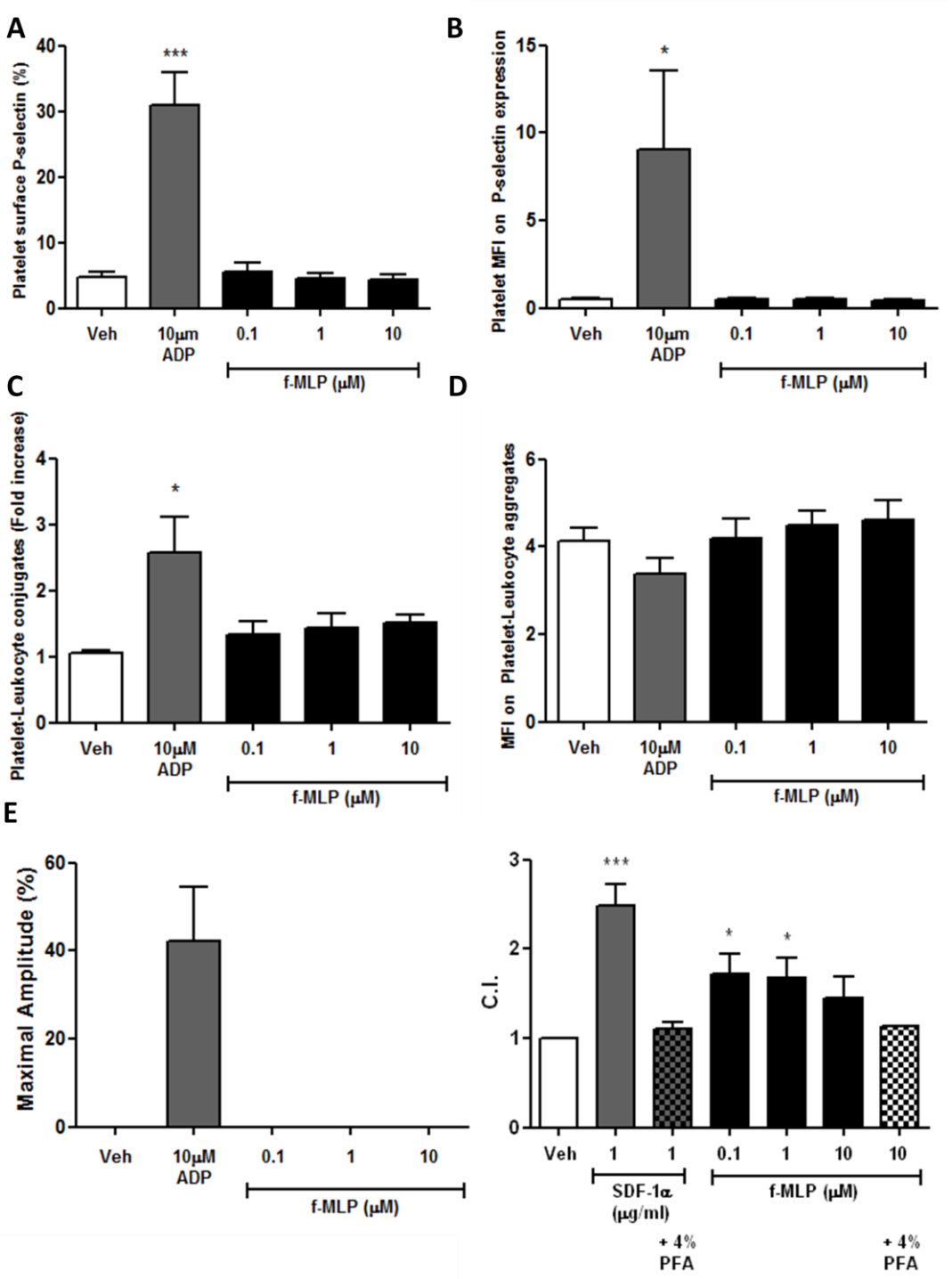


Figure 5. 3. Investigating the effects of the FPR receptor chemokine f-MLP on parameters of platelet activation

Stimulation with 0.1, 1 or 10µM f-MLP on gel filtered platelets isolated from human venous blood exposed to showed increased P-selectin activity (**A:** %, **B**: MFI), on platelet leukocyte conjugation in human venous whole blood (**C**: %, **D**: MFI), platelet aggregation in gel filtered platelets (**E**) and platelet migration with washed platelets isolated from human venous blood (**F**) Data: Mean ± SEM, N = 5. ***** = P < 0.05, ****** = P < 0.01**, ***** = P < 0.001 compared to vehicle controls.

Interlukin-1β (IL-1β) stimulates the IL-R1 receptor on platelets and has previously been shown to induce platelet activation *in vitro* (Brown et al., 2013).

Platelet stimulation with IL-1β failed to induce any increases in surface P-selectin expression *in vitro* compared to vehicle controls, both in terms of the percent of platelets expressing P-selectin (Veh: 3.47 ± 0.93% vs. 0.1µg/ml IL-1β: 3.49 ± 0.88%; 1µg/ml IL-1β: 3.88 ± 1.06% and 10µg/ml IL-1β: 3.60 ± 1.06%, **figure 5.4A**) or platelet MFI on P-selectin expression (Veh: 0.60 ± 0.07 vs. 0.1µg/ml IL-1β: 0.61 ± 0.08; 1µg/ml IL-1β: 0.63 ± 0.08 and 10µg/ml IL-1β: 0.62 ± 0.09, **figure 5.4B**).

Stimulation of citrated whole blood with IL-1β was also unable to induce significant elevation of platelet-leukocyte conjugate formation compared to vehicle controls, however a trend is visible suggesting that Il-1β induced a small but non-significant increase in the incidence of platelet leukocyte conjugates (Veh: 1.00 ± 0.35 vs. 0.1µg/ml IL-1β: 1.36 ± 0.48; 1µg/ml IL-1β: 1.56 ± 0.52 and 10µg/ml IL-1β: 1.15 ± 0.52, **figure 5.4C**), but not MFI on platelet-leukocyte conjugates (Veh: 3.92 ± 0.51 vs. 0.1µg/ml IL-1β: 4.29 ± 0.39; 1µg/ml IL-1β: 4.02 ± 0.19 and 10µg/ml IL-1β: 3.75 ± 0.41, **figure 5.4D**).

Platelet aggregation remained unaffected following stimulation of gel filtered isolated platelets with IL-1β when compared to vehicle controls (Veh: 13.03 ± 3.92% vs. 0.1µg/ml IL-1β: 14.00 ± 4.04%; 1µg/ml IL-1β: 24.67 ± 0.88% and 10µg/ml IL-1β: 24.50 ± 0.50, **figure 5.4E**).

Finally, washed platelets isolated from human venous blood failed to demonstrate any significant migration towards IL-1β when compared to vehicle controls (Veh: 1.00 ± 0.06 vs. 0.1µg/ml IL-1β: 0.38 ± 0.01; 1µg/ml IL-1β: 1.02 ± 0.31; 10µg/ml IL-1β: 1.18 ± 0.43; 10µg/ml IL-1β + PFA: 0.93 ± 0.27, **figure 5.4F**).

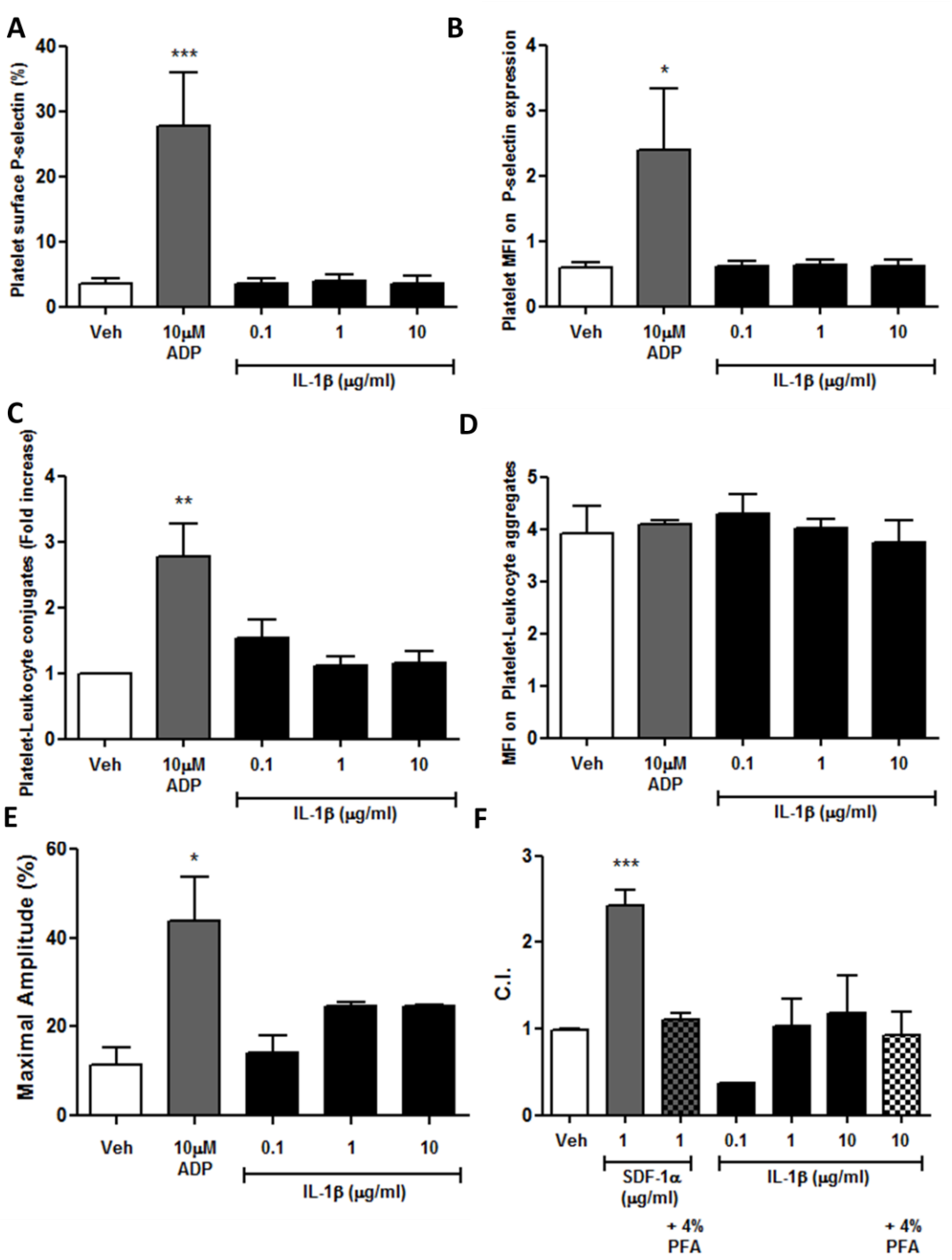


Figure 5. 4. Investigating the effects of the IL-R1 receptor chemokine IL-1β on parameters of platelet activation

Stimulation with 0.1, 1 or 10µg/ml IL-1β on gel filtered platelets isolated from human venous blood exposed to showed increased P-selectin activity (**A:** %, **B**: MFI), on platelet leukocyte conjugation in human venous whole blood (**C**: %, **D**: MFI), platelet aggregation in gel filtered platelets (**E**) and platelet migration with washed platelets isolated from human venous blood (**F**) Data: Mean ± SEM, N = 5. ***** = P < 0.05, ****** = P < 0.01**, ***** = P < 0.001 compared to vehicle controls.

5-HT stimulates the 5-HT2A receptor present on platelets, which has previously been described to induce platelet activation through a $G_{\alpha q}$ dependent mechanism. 5-HT stimulation was therefore investigated on a number of parameters of platelet activation. Platelet P-selectin expression was unaffected by 5-HT stimulation at all tested doses, both in terms of the incidence of platelets expressing P-selectin (Veh: 6.87 ± 2.22% vs. 0.1µg/ml 5-HT: 7.40 ± 3.52%; 1µg/ml 5-HT: 7.70 ± 2.73% and 10µg/ml 5-HT: 7.66 ± 2.77, **figure 5.5A**) and platelet MFI on P-selectin expression (Veh: 0.62 ± 0.08 vs. 0.1µg/ml 5-HT: 0.65 ± 0.09; 1µg/ml 5-HT: 0.61 ± 0.08 and 10µg/ml 5-HT: 0.62 ± 0.07, **figure 5.5B**).

The formation of platelet-leukocyte conjugates was also unaffected by stimulation of 5-HT at any tested dose in citrated whole blood compared to vehicle controls, both in the elevation of total platelet –leukocyte conjugates present (Veh: 1.00 ± 0.00 vs. 0.1µg/ml 5-HT: 1.38 ± 0.23; 1µg/ml 5-HT: 1.40 ± 0.23 and 10µg/ml 5-HT: 1.74 ± 0.34, **figure 5.5C**) and MFI on platelet-leukocyte conjugates (Veh: 3.92 ± 0.51 vs. 0.1µg/ml 5-HT: 4.48 ± 0.19; 1µg/ml 5-HT: 4.35 ± 0.21 and 10µg/ml 5-HT: 4.24 ± 0.17, **figure 5.5D**). Interestingly, whilst not significant a potential trend demonstrating a small increase was observed in the incidence of platelet-leukocyte conjugates but not MFI on platelet-leukocyte conjugates.

5-HT stimulation induced no platelet aggregation when compared with vehicle controls (Veh: 0.00 ± 0.00% vs. 0.1µg/ml 5-HT: 0.52 ± 0.52%; 1µg/ml 5-HT: 0.22 ± 0.22% and 10µg/ml 5-HT: 0.98 ± 0.48%, **figure 5.5E**), nor was there any observed increase in platelet migration compared to vehicle controls or stimulation of fixed platelets (Veh: 1.00 ± 0.00 vs. 0.1µg/ml 5-HT: 1.17 ± 0.25; 1µg/ml 5-HT: 1.18 ± 0.22; 10µg/ml 5-HT: 1.34 ± 0.36 and PFA treated 10µg/ml 5-HT: 0.96 ± 0.00, **figure 5.5F**).

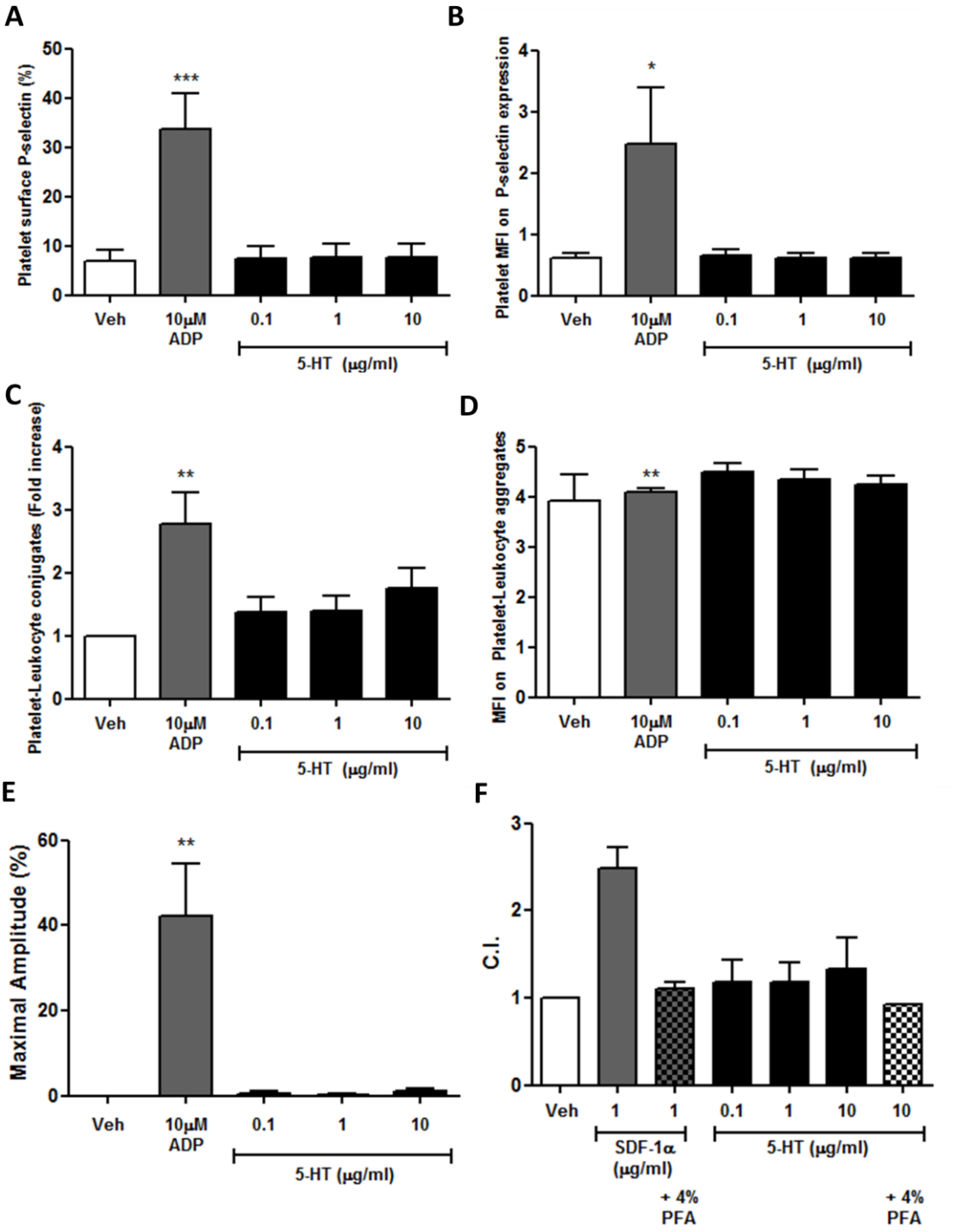


Figure 5. 5. Investigating the effects of 5-HT on parameters of platelet activation

Stimulation with 0.1, 1 or 10µg/ml 5-HT on gel filtered platelets isolated from human venous blood exposed to showed increased P-selectin activity (**A:** %, **B**: MFI), on platelet leukocyte conjugation in human venous whole blood (**C**: %, **D**: MFI), platelet aggregation in gel filtered platelets (**E**) and platelet migration with washed platelets isolated from human venous blood (**F**) Data: Mean ± SEM, N = 5. ***** = P < 0.05, ****** = P < 0.01**, ***** = P < 0.001 compared to vehicle controls.

Lipopolysaccharide (LPS), a pro-inflammatory glycol-lipid component of the bacterial cell wall of gram-negative bacteria has previously been described as a potent platelet agonist and has been shown to induce platelet activation through stimulation of platelet toll-like receptor 4 (TLR4) (Brown et al., 2013), therefore LPS stimulation was investigated on the panel of platelet activation assays.

Platelets stimulated with LPS *in vitro* failed to demonstrate any increase in P-selectin expression, in either the incidence of platelets expression P-selectin (Veh:6.24 ± 1.81% vs. 1µg/ml LPS: 6.37 ± 1.88%; 10µg/ml LPS: 7.28 ± 2.48% and 100µg/ml LPS: 7.32 ± 2.26%, **figure 5.6A**) or platelet MFI on P-selectin expression (Veh: 0.53 ± 0.10 vs. 1µg/ml LPS: 0.50 ± 0.07; 10µg/ml LPS: 0.51 ± 0.09 and 100µg/ml LPS: 0.53 ± 0.09, **figure 5.6B**).

LPS stimulation also failed to induce elevations in the formation of platelet-leukocyte conjugates in citrated whole blood, both in the incidence of the conjugates formation (Veh: 1.09 ± 0.06% vs. 1µg/ml LPS: 1.69 ± 0.21%; 10µg/ml LPS: 1.56 ± 0.31 and 100µg/ml LPS: 2.72 ± 0.08, **figure 5.6C**) and the MFI on platelet-leukocyte conjugates (Veh: 4.22 ± 0.52 vs. 1µg/ml LPS: 4.47 ± 0.68; 10µg/ml LPS: 4.38 ± 0.48 and 100µg/ml LPS: 3.88 ± 0.49, **figure 5.6D**), interestingly however, whilst the not significant a potential trend demonstrating an increase in the incidence of platelet leukocyte conjugates but not the MFI can be seen with increasing concentrations of LPS.

LPS stimulation of isolated gel filtered platelets failed to induce platelet aggregation compared to vehicle controls (Veh: 0.00 ± 0.00% vs. 1µg/ml LPS: 0.52 ± 0.52%; 10µg/ml LPS: 0.00 ± 0.00% and 100µg/ml LPS: 0.50 ± 0.50%, **figure 5.6E**). Similarly, platelets demonstrated no significant migration towards LPS at any tested

dose, indeed, 100µ/ml LPS appeared to attenuate basal levels of random platelet migration (Veh: 1.00 ± 0.00 vs. 1µg/ml LPS: 1.43 ± 0.27; 10µg/ml LPS: 1.20 ± 0.24; 100µg/ml LPS: 0.02 ± 0.01 and PFA treated 100µg/ml LPS: 0.67 ± 0.00, **figure 5.6F**).

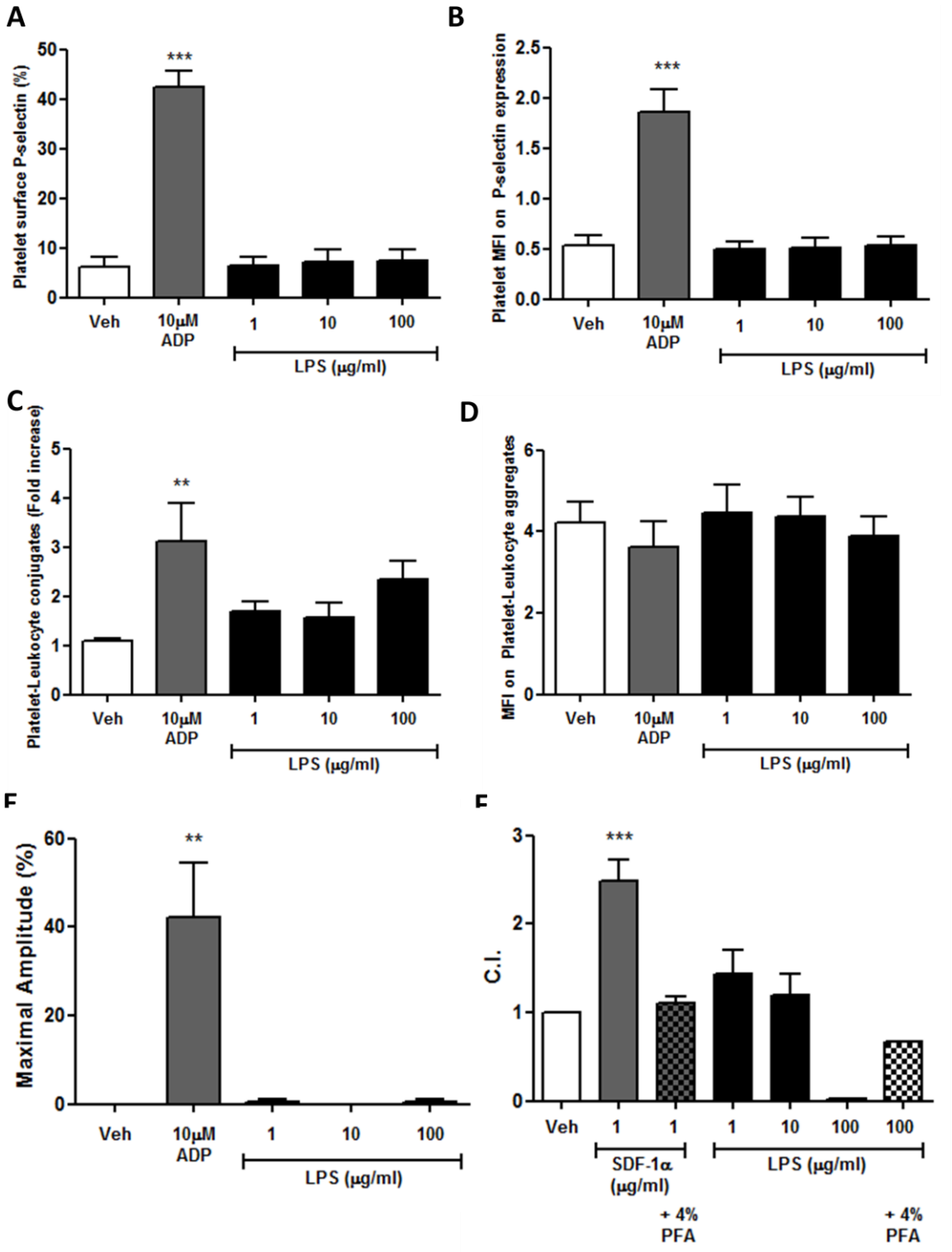


Figure 5. 6. Investigating the effects of LPS on parameters of platelet activation

Stimulation with 1, 10 or 100µg/ml LPS on gel filtered platelets isolated from human venous blood exposed to showed increased P-selectin activity (**A:** %, **B**: MFI), on platelet leukocyte conjugation in human venous whole blood (**C**: %, **D**: MFI), platelet aggregation in gel filtered platelets (**E**) and platelet migration with washed platelets isolated from human venous blood (**F**) Data: Mean ± SEM, N = 5. ***** = P < 0.05, ****** = P < 0.01**, ***** = P < 0.001 compared to vehicle controls.

5.3 An Investigation in to Effects of Classical Platelet Agonist Stimulation on Platelet Function

Adrenaline stimulates platelets via the α2A adrenoceptor coupled to Gαi, but has also been shown to stimulate platelets via the Gαs coupled β2 adrenoceptor (Benovic, 2002). The effects of adrenaline on the different paramaters of platelets were subsequently investigated.

Stimulation of isolated gel-filtered platelets induced an increase in P-selectin expression in both the incidence of platelets expressing P-selectin (Veh: 5.22 ± 1.38% vs. 1μM adrenaline: 4.68 ± 2.43%; 10μM adrenaline: 9.62 ± 2.82%; and 100μM adrenaline: 13.54 ± 3.34%, **figure 5.7A**) and platelet MFI on P-selectin expression (Veh: 0.62 ± 0.08; 1μM adrenaline: 0.75 ± 0.12; 10μM adrenaline: 0.85 ± 0.16 and 100μM adrenaline: 0.87 ± 0.34, **figure 5.7B**), however these elevations were not significantly different to vehicle levels.

Adrenaline stimulation also induced elevations in the formation of platelet-leukocyte conjugates in citrated whole blood, with increases observed in both the incidence of platelet leukocyte conjugates (Veh: 1.00 ± 0.00 vs. 1μM adrenaline: 1.55 ± 0.35%; 10μM adrenaline: 1.98 ± 0.43% and 100μM adrenaline: 2.24 ± 0.61, **figure 5.7C**) and MFI on platelet-leukocyte conjugates (Veh: 3.92 ± 0.51 vs. 1μM adrenaline: 3.56 ± 0.23; 10μM adrenaline: 4.49 ± 0.41 and 100μM adrenaline: 4.88 ± 0.36, **figure 5.7D**), although these elevations were not significant compared to vehicle controls.

Platelet stimulation with Adrenaline also induced platelet aggregation compared to vehicle control (Veh: 5.28 ± 2.89% vs. 1μM adrenaline: 2.43 ± 0.76%; 10μM adrenaline: 21.00 ± 10.78% and 100μM adrenaline: 21.32 ± 10.21%, **figure 4.7E**),

although aggregation induced by 100µM adrenaline was still substantially lower than aggregation induced by the positive control of 10µM ADP (42.20 ± 12.17%). No platelet migration was observed to adrenaline, with all tested concentrations remaining similar to vehicle and fixed platelet stimulated samples (Veh: 1.00 ± 0.00 vs. 1µM adrenaline: 1.06 ± 0.09; 10µM adrenaline: 1.18 ± 0.13; 100µM adrenaline: 1.03 ± 0.19 and PFA treated 100µM adrenaline: 1.08 ± 0.27, **figure 5.7F**).

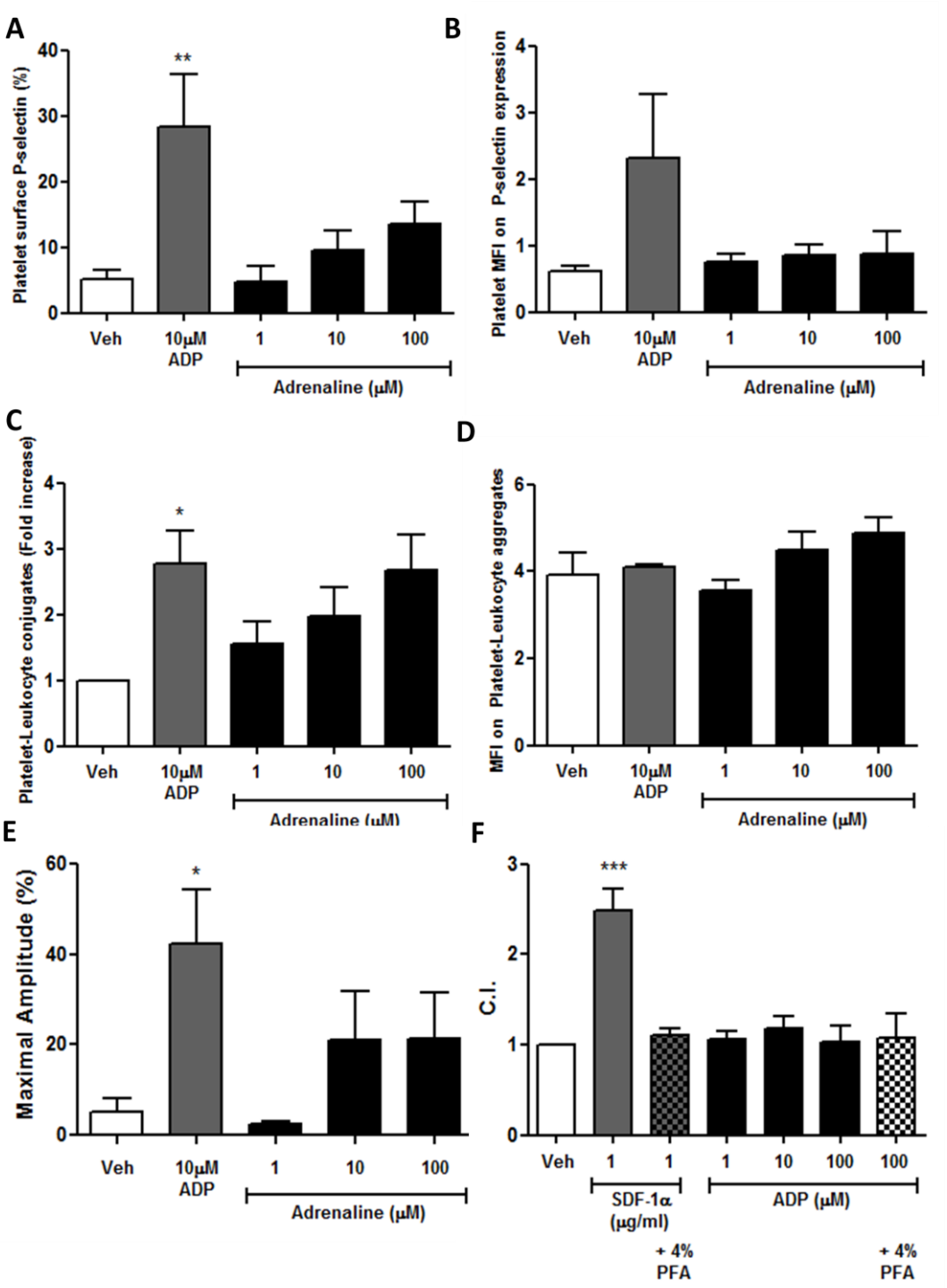


Figure 5. 7. Investigating the effects of adrenaline on parameters of platelet activation

Stimulation with 1, 10 or 100µM Adrenaline on gel filtered platelets isolated from human venous blood exposed to showed increased P-selectin activity (**A:** %, **B**: MFI), on platelet leukocyte conjugation in human venous whole blood (**C**: %, **D**: MFI), platelet aggregation in gel filtered platelets (**E**) and platelet migration with washed platelets isolated from human venous blood (**F**) Data: Mean ± SEM, N = 5. ***** = $P < 0.05$, ****** = $P < 0.01$, ******* = $P < 0.001$ compared to vehicle controls.

Stimulation of platelets with ADP results in the activation of both the $P2Y_1$ and $P2Y_{12}$ receptors, which when stimulated together triggers platelet granule release resulting in increases in expression of surface adhesion molecules, Ca^{2+} mobilisation, integrin $\alpha_{IIb}\beta_3$ activation, and platelet aggregation. The effects of ADP on the parameters of platelet activation were investigated.

Stimulation of platelets with ADP as expected induced a significant elevation in P-selectin expression, both in terms of the incidence of platelets expressing P-selectin (Veh: 3.52 ± 0.45% vs. 0.1µM ADP: 3.83 ± 0.41%; 1µM ADP: 10.62 ± 5.53% and 10µM ADP: 30.21 ± 5.32%, $P < 0.001$, **figure 5.8A**) and MFI on P-selectin expression (Veh: 0.51 ± 0.08 vs. 0.1µM ADP: 0.52 ± 0.08; 1µM ADP: 0.69 ± 0.09 and 10µM ADP: 1.59 ± 0.27, $P < 0.001$, **figure 5.8B**).

ADP stimulation also induced significant increases in the formation of platelet leukocyte conjugates, but only in terms of the incidence of platelet-leukocyte conjugates (Veh: 1.00 ± 0.45% vs. 0.1µM ADP: 1.28 ± 0.74%; 1µM ADP: 1.88 ± 1.08% and 10µM ADP: 2.83 ± 1.27%, $P < 0.05$, **figure 5.8C**) and not the MFI on platelet-leukocyte conjugates which remained similar to that seen in vehicle treated samples (Veh: 3.68 ± 0.65 vs. 0.1µM: 3.62 ± 0.61; 1µM: 3.28 ± 0.27 and 10µM ADP: 3.59 ± 0.70, **figure 5.8D**).

ADP stimulation also induced significant platelet aggregation compared to vehicle controls (Veh: 2.83 ± 1.74% vs. 0.1µM ADP: 2.65 ± 1.61%; 1µM ADP: 19.40 ± 13.41% and 10µM ADP: 42.20 ± 12.17%, $P < 0.01$, **figure 5.8E**), however no significant platelet migration was observed towards ADP at any tested concentration (Veh: 1.00 ± 0.00; 0.1µM ADP: 1.14 ± 0.06; 1µM ADP: 1.24 ± 0.23; 10µM ADP: 1.19 ± 0.21 and PFA treated 10µM ADP: 1.06 ± 0.21, **figure 5.8F**).

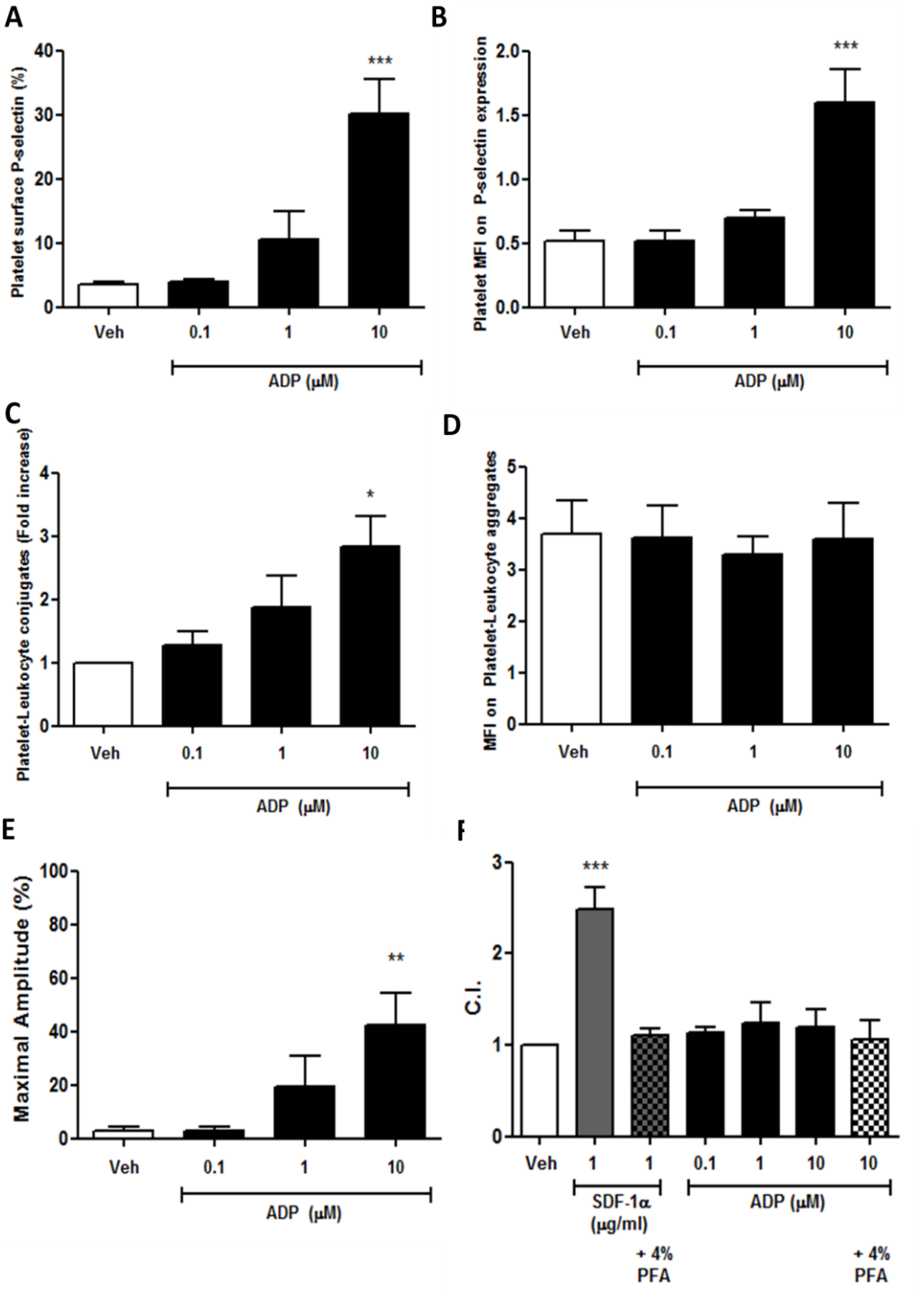


Figure 5. 8. Investigating the effects of the purinergic agonist ADP on parameters of platelet activation

Stimulation with 0.1, 1 or 10µM ADP on gel filtered platelets isolated from human venous blood exposed to showed increased P-selectin activity (**A:** %, **B**: MFI), on platelet leukocyte conjugation in human venous whole blood (**C**: %, **D**: MFI), platelet aggregation in gel filtered platelets (**E**) and platelet migration with washed platelets isolated from human venous blood (**F**) Data: Mean ± SEM, N = 5. ***** = P < 0.05, ****** = P < 0.01**, ***** = P < 0.001 compared to vehicle controls.

U46619 is a synthetic analogue of the platelet agonist thromboxane A2 known to stimulate platelet activation through the TP receptors on the platelets surface (Li et al., 2010). In this section the impact of TP receptor stimulation on a wide range of platelet activation parameters, both haemostatic and inflammatory were investigated.

Platelet stimulation with U46619 induced significant elevations in surface P-selectin expression, both in terms of the incidence of platelets expressing P-selectin (Veh: 8.94 ± 2.06% vs. 10nM U46619: 7.52 ± 2.09%; 100nM U46619: 18.93 ± 0.65% and 1000nM U46619nM: 53.70 ± 5.40%, $P < 0.001$, **figure 5.9A**) and platelet MFI on P-selectin expression (Veh: 0.67 ± 0.09 vs. 10nM U46619: 0.65 ± 0.12; 100nM U46619: 1.04 ± 0.12 and 1000nM U46619: 3.93 ± 0.65, $P < 0.001$, **figure 5.9B**).

U46619 stimulation also resulted in significant increases in the formation of platelet-leukocyte conjugates, however increases were only observed in the incidence of platelet leukocyte conjugates (Veh: 1.00 ± 0.00% vs. 10nM U46619: 1.11 ± 0.16%; 100nM U46619: 1.32 ± 0.19% and 1000nM U46619: 2.75 ± 0.26%, $P < 0.001$, **figure 5.9C**) and not MFI on platelet-leukocyte conjugates (Veh: 3.53 ± 0.74 vs. 10nM U46619: 2.82 ± 0.47; 100nM U46619: 2.80 ± 0.34 and 1000nM U46619: 3.02 ± 0.74, **figure 5.9D**).

In addition, platelet stimulation with U46619 induced substantial platelet aggregation compared to vehicle controls (Veh: 2.04 ± 1.59%; 10nM U46619: 5.50 ± 4.27%; 100nM U46619: 52.09 ± 9.31%, $P < 0.001$ and 1000nM U46619: 81.76 ± 8.97%, $P < 0.001$, **figure 5.9E**), with significantly elevated aggregation observed towards 1000nM U46619 compared to the 10µM ADP positive control (10µM ADP: 42.20 ± 12.17% vs. 1000nM U46619: 1000nM U46619: 81.76 ± 8.97%, $P < 0.001$).

No significant migration was observed towards U46619 at any tested dose, with levels of migration remaining within ranges observed for vehicle control and stimulation of fixed platelets (Veh: 1.00 ± 0.00 vs. 10nM U46619: 1.24 ± 0.14; 100nM: 1.08 ± 0.19; 1000nM U46619: 1.23 ± 0.20 and PFA treated 1000nM U46619: 1.15 ± 0.22, **figure 5.9F**).

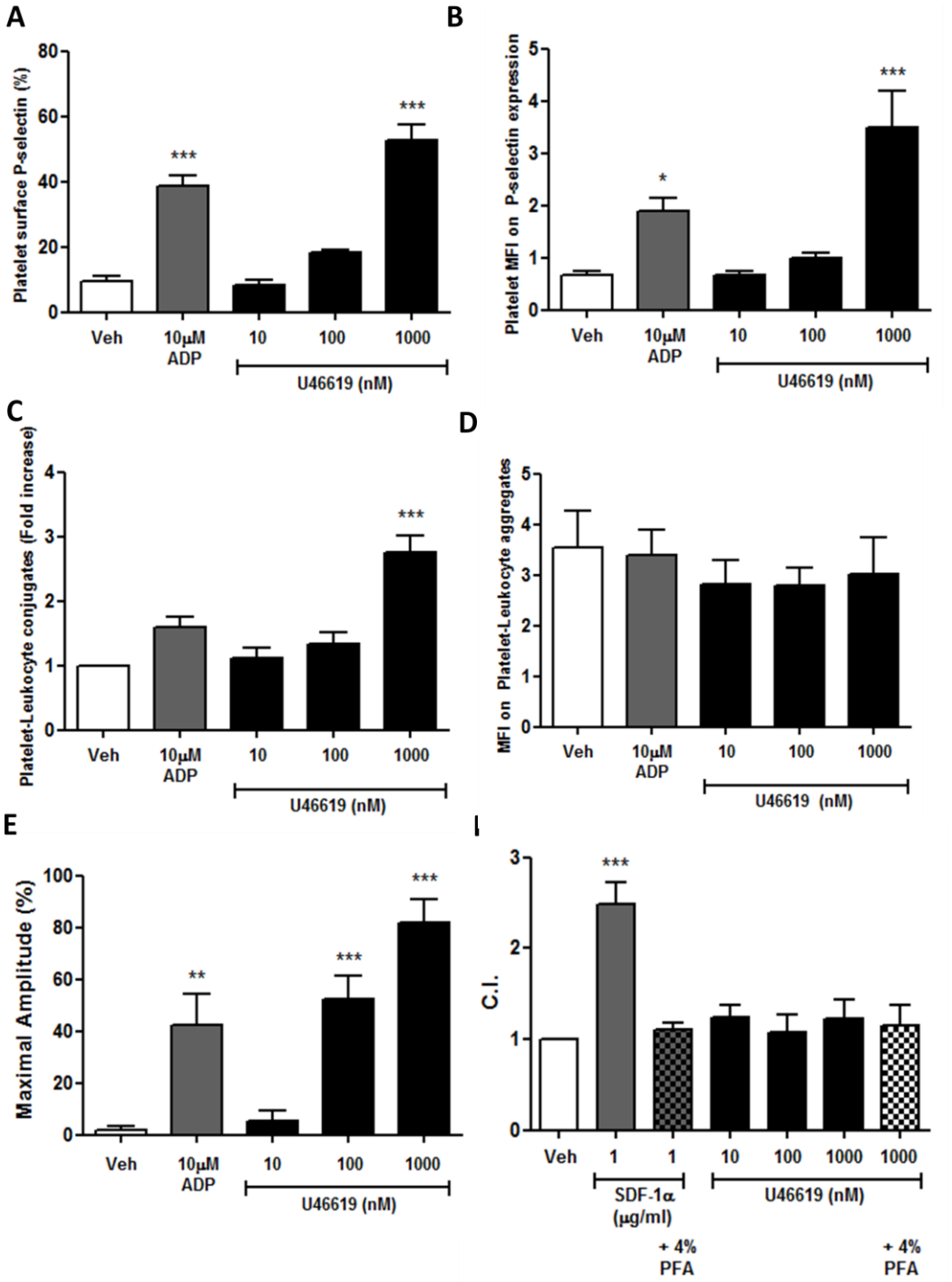


Figure 5. 9. Investigating the effects of the TxA_2 analogue U46619 on parameters of platelet activation

Stimulation with 10, 100 or 1000nM U46619 on gel filtered platelets isolated from human venous blood exposed to showed increased P-selectin activity (**A:** %, **B**: MFI), on platelet leukocyte conjugation in human venous whole blood (**C**: %, **D**: MFI), platelet aggregation in gel filtered platelets (**E**) and platelet migration with washed platelets isolated from human venous blood (**F**) Data: Mean ± SEM, N = 5. ***** = $P < 0.05$, ****** = $P < 0.01$**,** ******* = $P < 0.001$ compared to vehicle controls.

Thrombin receptor activating peptide (TRAP) is a platelet agonist stimulating platelet aggregation through stimulation of the $G_{\alpha q}$ coupled PAR receptors (Soh et al., 2010). TRAP induced platelet activation was investigated further through the use of both haemostatic and inflammatory assays of platelet activation.

As expected, platelet stimulation with TRAP resulted in a significant increase in both the incidence of platelets expressing P-selectin (Veh: 7.58 ± 2.67% vs. 0.1µM TRAP: 13.30 ± 6.53%; 1µM TRAP: 27.91 ± 5.74% and 10µM TRAP: 78.13 ± 7.18, $P < 0.001$, **figure 5.10A**) and platelet MFI on P-selectin expression (Veh: 0.64 ± 0.13 vs. 0.1µM TRAP: 0.60 ± 0.08; 1µM TRAP: 0.71 ± 0.15 and 10µM TRAP: 9.42 ± 1.99, $p < 0.001$, **figure 5.10B**) compared to vehicle controls.

Furthermore, stimulation of citrated whole blood induced a significant increase in the formation of platelet-leukocyte conjugates (Veh: 1.00 ± 0.00% vs. 0.1µM TRAP: 1.31 ± 0.16%; 1µM TRAP: 1.49 ± 0.52% and 10µM TRAP: 3.05 ± 0.78%, $P < 0.01$, **figure 4.10C**) but not MFI on platelet-leukocyte conjugates (Veh: 3.68 ± 1.65 vs. 0.1µM TRAP: 3.24 ± 1.45; 1µM TRAP: 3.61 ± 1.62 and 10µM TRAP: 3.85 ± 1.74, **figure 5.10D**).

Platelet aggregation was significantly elevated following stimulation with increasing concentrations of TRAP compared to vehicle controls (Veh: 3.31 ± 2.54% vs. 0.1µM TRAP: 1.41 ± 1.11%; 1µM TRAP: 33.41 ± 10.11%, $P < 0.05$ and 10µM TRAP: 69.35 ± 8.54%, $P < 0.001$, **figure 4.10E**), with 10µM TRAP inducing significantly higher levels of platelet aggregation compared to aggregation induced by 10µM ADP as the positive control (10µM ADP: 42.20 ± 12.17% vs. 10µM TRAP: 69.35 ± 8.35%, $P < 0.001$).

Finally, no platelet migration was observed to TRAP at any tested concentration (Veh: 1.00 ± 0.00 vs. 0.1µM TRAP: 1.25 ± 0.24; 1µM TRAP: 1.10 ± 0.24; 10µM TRAP: 1.10 ± 0.19 and PFA treated 10µM TRAP: 1.01 ± 0.17, **figure 5.10F**).

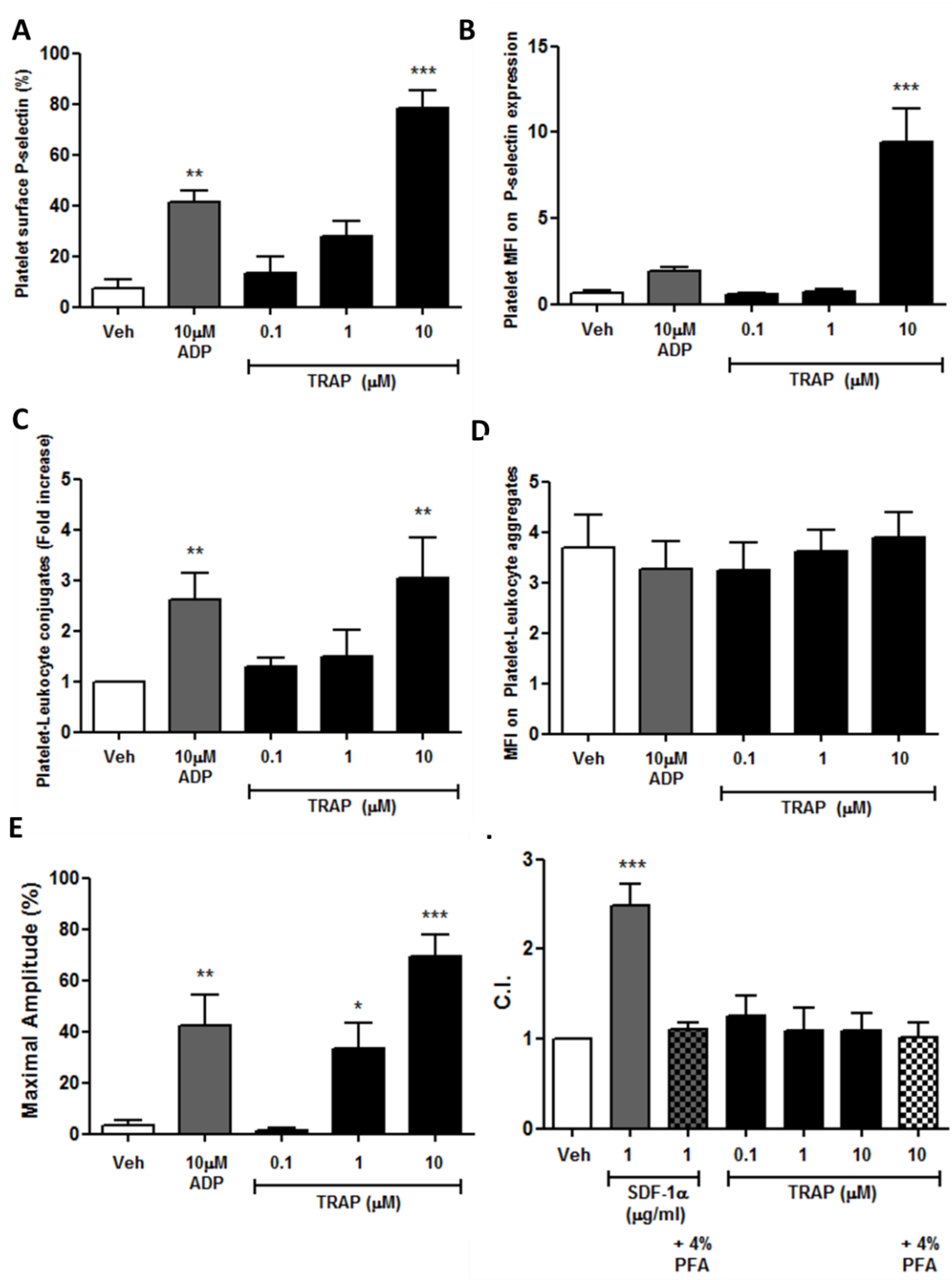


Figure 5. 10. Investigating the effects of the PAR receptor agonist TRAP on parameters of platelet activation

Stimulation with 0.1, 1 or 10µM TRAP on gel filtered platelets isolated from human venous blood exposed to showed increased P-selectin activity (**A:** %, **B**: MFI), on platelet leukocyte conjugation in human venous whole blood (**C**: %, **D**: MFI), platelet aggregation in gel filtered platelets (**E**) and platelet migration with washed platelets isolated from human venous blood (**F**) Data: Mean ± SEM, N = 5. ***** = $P < 0.05$, ****** = $P < 0.01$**,** ******* = $P < 0.001$ compared to vehicle controls.

5.4 A Summary of the Dichotomy in Platelet Function

As the previous studies have demonstrated, the 3 inflammatory chemokines SDF-1α, MDC and f-MLP demonstrated significant increases in platelet chemotactic ability, however no significant change was observed in other platelet activation markers such as P-selectin expression, Platelet-leukocyte conjugation or platelet aggregation. In contrast the classical platelet agonists including TxA_2, ADP, adrenaline and TRAP all demonstrated elevations in P-selectin expression, Platelet-Leukocyte conjugation and platelet aggregation.

Interestingly 5-HT, IL-1β and LPS all failed to produce signs of platelet activation in any of the tested parameters. The complete data set is summarised below in table 4.1.

Stimuli	Platelet P-selectin	Platelet-Leukocyte aggregates	Platelet Aggregation	Platelet Migration
SDF-1				√
MDC				√
f-MLP				√
IL-1β		X		
5-HT		X		
LPS		X		
Adrenaline	√	√	√	
ADP	√	√	√	
U46	√	√	√	
TRAP	√	√	√	

Table 4. Summarising a dichotomy in platelet function
√ = significant increases, x = increases but not significant

The inability of the chemokines SDF-1α and MDC as well as the other inflammatory stimuli to modulate typical platelet function was surprising, but a explanation may rely in observed synergism between chemokine stimulation and sub-threshold doses of ADP(Gear, 2001; Suttitanamongkol and Gear, 2001).

5.5 An Investigation into the Potential Potentiation of ADP induced Platelet Activation with MDC Co-Stimulation

Under normal conditions as it is demonstrated that primary agonists induce platelet activation as seen through platelet aggregation, P-selectin and Platelet-Leukocyte aggregate experiments, whilst other agonists such as adrenaline can play important amplify agonist induced platelet aggregation. Given that platelets expresses the MDC chemokine receptor CCR4, combined with the previously described platelet induced PMN chemotaxis assays, it stood to reason that MDC may be capable of inducing platelet activation or at least modulate ADP induced platelet activation. The ability of MDC to either directly induce platelet activation or modulate ADP induced platelet activation through an *in vitro* platelet-leukocyte aggregate assay was therefore investigated. Washed platelets were isolated from citrated blood collected via cardiac puncture and subsequently stimulated with MDC, ADP or a co-stimulation of both MDC and ADP. Following stimulation, washed platelets were mixed with bone marrow derived PMN cells and platelet-PMN aggregates measured by flow cytometry as a measure of platelet activation (figure 3.45).

Platelets stimulated with 10μM ADP induced significant formation of platelet-PMN aggregates (58.02 ± 9.80% above basal, $P < 0.001$). In contrast, stimulation with 0.1μM MDC failed to induce any significant elevation of platelet-PMN conjugation above basal levels (Basal: 0.00 ± 3.66% vs. 4.04 ± 3.76%). Co-stimulation of

platelets with both 10µM ADP and 0.1µM MDC further enhanced platelet-PMN aggregate formation with a 21.8% increase in platelet-aggregates observed in co-stimulated samples compared to ADP stimulation alone (79.79 ± 5.12%, $P < 0.05$), indicating that whilst stimulation with MDC alone was insufficient to induce platelet activation, in combination with a primary agonist it was capable of potentiating platelet activation, which is in agreement with published work (Clemetson et al., 2000; Gear, 2001).

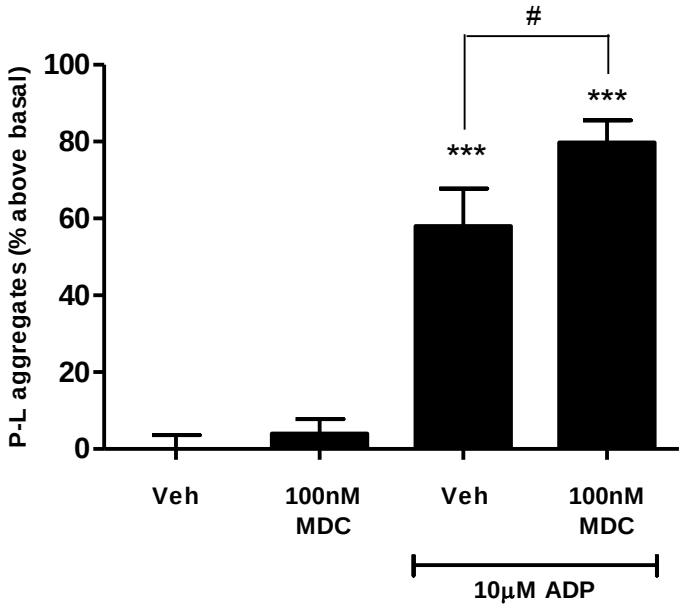


Figure 5. 11. Potential MDC potentiation of ADP induced platelet-leukocyte conjugate formation

Platelets and PMNs were isolated from citrated blood and bone marrow from female Balb/c mice. Platelets were stimulated with 0.1µM MDC, 10µM ADP or co stimulation with both 0.1µM MDC and 10µM ADP before mixing with PMN cells. Platelet-leukocyte conjugation was subsequently measured via flow cytometry. Data expressed as mean ± SEM of % above basal. * = $P < 0.05$, *** = $P < 0.001$. # = $P < 0.05$ where indicated n = 5.

Chapter VI

Discussion

6.1 The Role of Purines in an Acute Model of Allergic Inflammation

The role of purines and the platelet purinergic receptors in platelet activation has been extensively documented. Stimulation of platelet $P2Y_1$ and $P2Y_{12}$ receptors has been observed to induce increases in P-selectin expression whilst the $P2X_1$ receptor involvement has been implicated in platelet shape change. All 3 purinergic receptors are implicated in platelet aggregation (Sun et al., 1998; Storey et al., 2000, 2002; Leon et al., 2003; Hechler et al., 2005; Gachet, 2006; Mahaut-Smith et al., 2011). Furthermore, recent research has implicated platelet purinergic receptor stimulation in allergic inflammation (Idzko et al., 2007; Paruchuri et al., 2009). Whilst currently, the importance of purines in platelet activation in the context of allergic inflammation remains unclear, it is becoming an area of increasing interest. Indeed at the time of writing a clinical trial has commenced investigating the impact of clopidogrel treatment on sputum eosinophil levels following allergen challenge in patients with asthma (http://clinicaltrials.gov/ct2/show/record/NCT01955512). In this thesis, evidence of a distinct mechanism of platelet activation within the context of allergic inflammation leading to pulmonary leukocyte recruitment is provided.

Initial experiments were performed using the enzyme apyrase responsible for hydrolysing active ATP & ADP into inactive AMP. Treatment of mice sensitised and challenged to the allergen OVA with 100U/ml apyrase intravenously demonstrated significant attenuation of pulmonary macrophage, neutrophil, lymphocyte and eosinophil recruitment, thus agreeing with work demonstrating inhibition of neutrophil chemotaxis *in vitro* following apyrase treatment (Chen et al., 2006). This suggests a role of purine stimulation in the context of allergic inflammation. Whilst this study implicates purines generally, later experiments specifically investigated the role of the 3 purinergic receptors expressed on the

surface of platelets ($P2X_1$, $P2Y_1$ and $P2Y_{12}$). Whilst administration of the $P2X_1$ receptor specific suramin analogue NF-279 failed to inhibit allergen induced pulmonary leukocyte recruitment, treatment with both the $P2Y_1$ specific receptor antagonists MRS2179 and MRS2500 significantly attenuated pulmonary leukocyte recruitment as measured in BALF in a dose-dependent manner. Interestingly, $P2Y_{12}$ receptor antagonism with MRS2395, AR-C66096 or Clopidogrel demonstrated no attenuation of allergen induced pulmonary leukocyte recruitment at any tested dosage. We confirmed that, the inability of the $P2Y_{12}$ receptor antagonists was not a result of insufficient biological activity in this model as the highest doses of both AR-C66096 and Clopidogrel continued to suppress platelet aggregation towards ADP 6 hours post allergen challenge *ex vivo*. Elevated numbers of circulating platelet-leukocyte conjugates have previously been demonstrated in asthmatic patients following allergen provocation as well as within the murine model of allergic inflammation used in these investigations (Gresele et al., 1993; Pitchford et al., 2003a, 2005; Kowal et al., 2006; Johansson et al., 2012), with these circulating complexes believed to 'prime' leukocytes for efficient adhesion the vascular endothelium prior to transmigration into inflamed tissue. In this investigation, antigen exposure demonstrated significant elevations in circulating platelet-leukocyte complexes at 6 hours post allergen challenge, with levels returning to basal levels at 24 hours, in agreement with the literature. $P2Y_1$ receptor antagonist treatment ($P2Y_1$) suppressed allergen induced elevations in conjugate formation at 6 hours; however this was not replicated by $P2Y_{12}$ receptor antagonist treatment (AR-C66096). This combined with pulmonary leukocyte recruitment the data suggests that platelet activation during the inflammatory response to allergen is mediated via the $P2Y_1$ receptor.

Given the accepted role of the $P2Y_{12}$ receptor in platelet aggregation to all platelet activating stimuli (Gachet, 2006), the failure of $P2Y_{12}$ receptor antagonism to modulate allergen induced leukocyte recruitment or the formation of platelet-leukocyte conjugates came as a surprise. Indeed, this is of particular interest given that previous studies have demonstrated decreased formation of platelet-leukocyte conjugates and P-selectin expression in Clopidogrel treated mice (Evangelista et al., 2005) and their importance in leukocyte recruitment in numerous inflammatory diseases (Hayward et al., 1999; Jawieñ et al., 2002; Pitchford et al., 2005; Zarbock et al., 2007; Kornerup et al., 2010). Whilst in these studies $P2Y_{12}$ receptor antagonism was not involved in pulmonary leukocyte recruitment and failed to attenuate allergen induced elevations in circulating platelet-leukocyte conjugates, other laboratories have demonstrated conflicting impacts of the $P2Y_{12}$ receptor in inflammatory disorders with both protective (Garcia et al., 2011; Liverani et al., 2014) and potentiating roles (Paruchuri et al., 2009) observed in inflammatory cell recruitment. It is possible that the conflicting roles of $P2Y_{12}$ receptors may be dependent on the provocation stimuli used in each study. In this model platelets were activated via an IgE-dependent mechanism following antigen (OVA) challenge in contrast to direct stimulation of platelets by leukotrienes (LTD_4/LTE_4) or Lipopolysaccharide (LPS) that has been presented in alternate models (Paruchuri et al., 2009; Liverani et al., 2014).

Platelet activation of purinergic receptors under haemostatic conditions is typically instigated by initial activation of the high affinity platelet $P2Y_1$ ADP receptor promoting internal Ca^{2+} mobilisation and α-granule secretion. This leads to platelet derived ADP release triggering subsequent activation of the lower affinity $P2Y_{12}$ ADP receptor (Jin et al., 1998; Fabre et al., 1999; Kunapuli, 2003). This secondary

activation of $P2Y_{12}$ receptors then promotes dense granule secretion inducing local generation of thrombin and TxA_2 promoting integrin activation and platelet aggregation (Storey et al., 2000; Kauffenstein et al., 2001). With the understanding that under haemostatic conditions $P2Y_1$ activation triggers subsequent $P2Y_{12}$ activation promoting platelet aggregation, it remains unclear as to why platelet activation by inflammatory mediators promoting leukocyte recruitment via $P2Y_1$ receptor stimulation does not ultimately lead to thrombosis and coagulation *in vivo.* One hypothesis is that following allergen sensitisation, alterations in the surface expression of the purinergic receptors might occur. However, studies investigating $P2Y_1$ and $P2Y_{12}$ receptor expression post allergen challenge demonstrated no modulation in either receptors' expression profile. The $P2Y_1$ and $P2Y_{12}$ receptors demonstrate different affinities for ADP, with the $P2Y_1$ receptor demonstrating a much higher affinity compared to the P2Y12 receptor (Kunapuli, 2003). It is therefore possible that in regions under inflammatory stress the local concentration of ADP is lower when compared to regions of thrombosis which are unable to induce $P2Y_{12}$ receptor activation.

As the formation of platelet-leukocyte complexes post allergen challenge is P-selectin dependent (Hayward et al., 1999; Jawieñ et al., 2002; Schober, 2002; Pitchford et al., 2005; Zarbock et al., 2006; Kornerup et al., 2010) and is affected by $P2Y_1$ receptor signalling it was possible that the dependency on $P2Y_1$ receptor activation mediating engagements with leukocytes *in vivo* might stimulate the expression of leukocyte adhesion molecules enhancing leukocyte transmigration into inflamed tissue. To measure this a functional assay of platelet induced PMN cell migration previously described by Kornerup et al., 2010 towards a MDC, SDF-1α and f-MLP and its dependency on P-selectin, and the impact of the purinergic

receptors was investigated following stimulation with low dose (sub-threshold for aggregation, 100nM) ADP. This potentially indentifies a source of cross-over signalling between separate GPCRs (i.e. CXCR4 and $P2Y_1$ or CCR4 and $P2Y_1$), allowing ADP at concentrations below the threshold for the initiation of platelet aggregation (100nM) to trigger platelet activation and stimulate the migration of leukocytes towards a chemotactic stimulus. Indeed, MDC is known to activate platelets in an ADP-dependent manner and MDC has previously been demonstrated to be present in elevated levels in asthmatic patients (Gear, 2001; Hirata et al., 2003).

This potential cross-talk between receptors is particularly important given production of second messengers can be amplified by coincidental receptor activation. Cross-talk between the G_i and G_q signalling pathways can potentiate smooth muscle contraction when stimulated by low (almost threshold) concentrations of agonists (Selbie and Hill, 1998), whilst other studies have shown cross-talk between the G_q linked $P2Y_1$ and the G_i linked $P2Y_{12}$ receptor in Ca^{2+} mobilisation and PI3-K activity (Hardy et al., 2004). This mechanism of receptor cross-talk potentially demonstrates a mechanism by which chemokines such as MDC and SDF-1α can induce platelet activation following co-stimulation with ADP and sub-threshold concentrations.

Experiments confirmed that platelets were critical for PMN cell migration towards both MDC and SDF-1. Furthermore, platelet-induced PMN migration was significantly attenuated by pre-incubation of platelets with antagonists of either the CCR4 (AMD3100) or CXCR4 (C-021) receptors which are known to be expressed on the platelets surface antagonists (Clemetson et al., 2000), thus confirming that direct chemokine stimulation of platelets was critical in the instigation of PMN cell migration. The assay was also confirmed to be dependent upon P-selectin interactions with PSGL-1 through the use of an anti—P-selectin antibody proving its

relevance to the *in vivo* P-selectin dependent model of pulmonary leukocyte recruitment (Pitchford et al., 2003a, 2005).

Sensitisation to OVA demonstrated significant increases in platelet induced PMN chemotaxis when compared to sham controls. This suggests that allergen sensitised platelets potentially become 'primed' for more efficient activation. Indeed, previous studies have demonstrated platelets are amongst a number of cells capable of being activated by allergen, with subsequent increases in cell motility and migration observed (Pitchford et al., 2008; Kuehn et al., 2010). Furthermore, allergen challenge resulted in a mild but significant state of systemic thrombocytopenia between 3 and 24 hours, presumably as a result of the migration of platelets attached to leukocytes into the tissue but also potentially through direct recruitment and tissue accumulation (Pitchford et al., 2008). Whilst re-infusion of thrombocytopenic mice with platelets isolated from allergen sensitised mice demonstrated greater restoration of allergen induced pulmonary eosinophilia when compared to platelets isolated from naive mice (Pitchford et al., 2003a). This potentially describes a mechanism by which allergen can 'prime' platelets for efficient interactions with leukocytes contributing to elevated PMN migration in this assay.

Pre-incubation of platelets with the $P2Y_1$ receptor antagonists MRS2179 or MRS2500 significantly inhibited PMN chemotaxis in a dose-dependent manner to both MDC and f-MLP, however neither the $P2Y_{12}$ receptor antagonists MRS2395 or AR-C66096 nor the $P2X_1$ receptor antagonists inhibited platelet-induced PMN chemotaxis towards any measured chemokine. Interestingly, the $P2Y_1$ antagonists failed to inhibit platelet-induced PMN chemotaxis towards SDF-1α, suggesting that PMN chemotaxis towards SDF-1α was independent of ADP, whilst platelet

activation via f-MLP and MDC initiated PMN migration in an ADP dependent manner.

$P2Y_1$ receptor expression is not solely restricted to platelets and has also been measured on epithelial, endothelial, immune cells and osteoclasts (Burnstock, 2007). Given the apparent regulatory role in P-selectin dependent PMN migration *in vitro,* the formation of platelet-leukocyte conjugates *ex vivo* post allergen challenge and pulmonary leukocyte recruitment *in vivo,* it was determined that the requirement of $P2Y_1$ receptors for pulmonary leukocyte recruitment was platelet specific and not through activation of systemic $P2Y_1$ receptors. To investigate the requirement of platelet specific $P2Y_1$ receptors, OVA sensitised mice were rendered thrombocytopenic through a previously documented dosing regimen with the bone marrow depleting toxin Busulfan (Pitchford et al., 2003a, 2004, 2005, 2008).

Thrombocytopenia can be induced using both immune and non-immune techniques, through either treatment with the bone marrow depleting toxin Busulfan, or through administration of anti-platelet antibodies i.e. APAS. For the purpose of these investigations, thrombocytopenia was induced through Busulfan treatment, as this enabled the investigation of the specific platelet purinergic receptors through reinfusion of drug treated platelets. In this investigation, 3 separate administrations of 20mg/kg Busulfan induced a severe state of thrombocytopenia characterised by an 80% decrease in circulating platelets. Importantly, the busulfan dosing profile did not demonstrate any toxicity towards leukocytes and no significant decreases in circulating leukocytes were observed. Furthermore, previous studies have shown that busulfan treatment had no effect on IgE titres in allergen sensitised mice following acute or chronic treatment (Pitchford et al., 2003a, 2004). Neither have any effects been observed on the production of the Th2 cytokines IL-4 and IL-5 which are

critical for the development of the allergic response in this model (Pitchford, 2003). Moreover, any non-specific actions of busulfan treatment can be ruled out through the restoration of the inflammatory response following platelet replenishment of thrombocytopenic mice. These investigations demonstrated attenuated allergen induced pulmonary leukocyte recruitment in busulfan treated mice, combined with a restoration of leukocyte recruitment following platelet replenishment in agreement with previous studies (Pitchford et al., 2003a, 2005). Replenishment of platelets isolated from donor mice treated with saturating concentrations of the $P2Y_1$ antagonist MRS2500 continued to result in significant suppression of pulmonary leukocyte recruitment, whilst reinfusion of AR-C66096 treated platelets demonstrated full restoration of allergen induced pulmonary leukocyte recruitment. This data combined with the previous experiments provides conclusive evidence of a role of the platelet specific $P2Y_1$ receptor in propagating pulmonary leukocyte recruitment but not the $P2Y_{12}$ receptor.

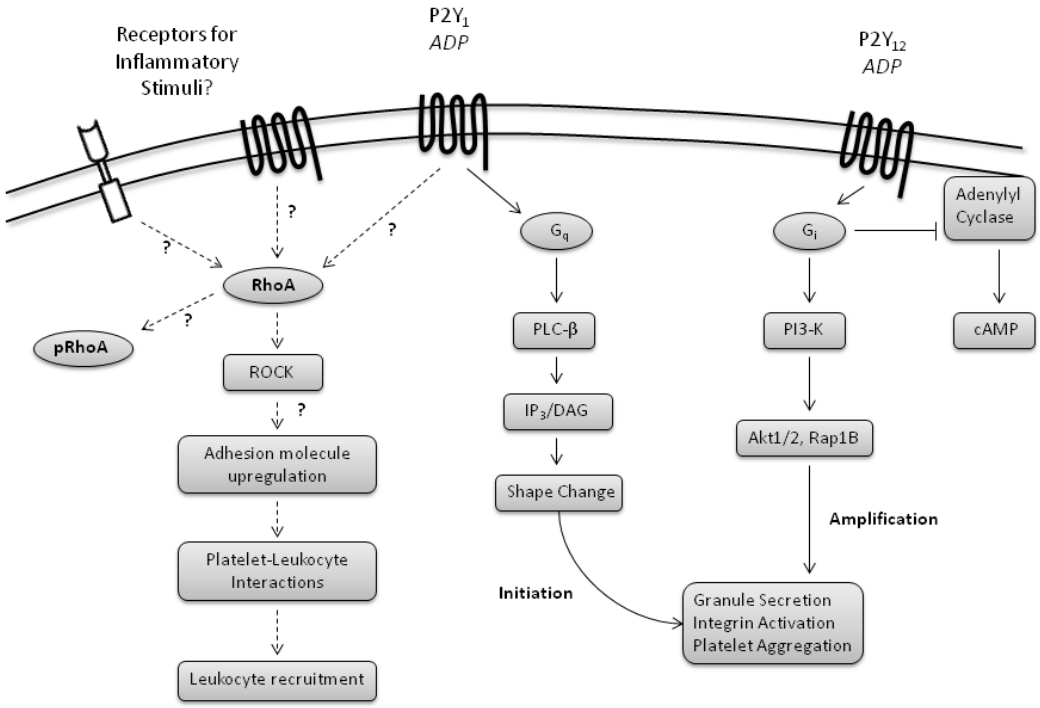


Figure 6. 1. A Potential dichotomy in platelet function through the $P2Y_1$ receptor

It remains unclear as to why platelet activation of the $P2Y_1$ receptor does not lead to subsequent activation of the $P2Y_{12}$ receptor promoting aggregation or coagulation. Indeed, rather than showing increased platelet aggregation asthmatic patients display a mild haemostatic defect manifested by increased bleeding times (Szczeklik et al., 1986; Ind, 1991). As previously discussed, normal activation of the $P2Y_{12}$ receptor signals through its associated G_i G protein inhibiting adenylyl cyclase activation through the α subunit decreasing cAMP production (Leon et al., 2003) or promoting dense granule secretion through phosphatidylinositol-3-kinase (PI3-K) activation mediated by the β/γ subunits (Hardy et al., 2004; Gachet, 2006). In contrast, the $P2Y_1$ receptor activation initiates signalling through its associated G_α G protein, typically signalling through PLCβ triggering Ca^{2+} mobilisation and PKC activation critical in the instigation of platelet shape change, granule secretion and initiating

aggregation (Hardy et al., 2004; Gachet, 2006). Interestingly however, the $P2Y_1$ receptor also appears to be able to couple to and activate the small GTPase RhoA which in other cells has been demonstrated to be involved in cell polarity and chemotaxis (Sakumura et al., 2005; Mong and Wang, 2009; Palani et al., 2012; Pleines et al., 2012; Aslan and McCarty, 2013; Sauzeau et al., 2013). The widely accepted role of the Rho family of GTP binding proteins is that of signalling switches through the hydrolysis of GTP to GDP. Small GTPase's mediation of signal transduction is homologous to the G_α subunits of heterotrimeric G proteins. In platelets they are responsible for the amplification of signals from platelet surface receptors. The 3 key members of the Rho GTPase family are RhoA, cdc42 and Rac1 (Aslan and McCarty, 2013), all of which have emerged as major regulators of actin cytoskeleton dynamics, platelet aggregation, granular secretion, spreading and thrombus formation (Aslan and McCarty, 2013; Ridley, 2013a). In its active form, RhoA mediates the activation of Rho Kinase (ROCK). Two ROCK isoforms exist, ROCK1 and ROCK2 sharing an overal 64% sequence identity with 89% identity in the catalytic domain on the N terminus with a functional redundancy observed between the 2 isoforms (Mong and Wang, 2009). RhoA can mediate its effects through either $G_{\alpha 13}$ or $G_{\alpha q}$. Through $G_{\alpha 13}$ stimulation, RhoA mediated activation of ROCK inactivates myosin light chain (MLC) phosphatase increasing MLC phosphorylation. This triggers subsequent actomyosin contractions and promotes platelet shape change. Alternatively, $G_{\alpha q}$ signalling can directly activate MLC kinase activation promoting direct phosphorylation of MLC promoting shape change and centralisation of secretory granules. (Mong and Wang, 2009; Pleines et al., 2012; Aslan and McCarty, 2013).

In this murine model of allergic inflammation, platelet activation downstream of $P2Y_1$ but not $P2Y_{12}$ was demonstrated to phosphorylate RhoA at residue Ser188 6 hours post allergen challenge *in vivo.* Furthermore, through inhibition of Rho associated kinase (ROCK) with the specific receptor antagonist GSK429286 and the RhoA inhibitor C3 exoenzyme, RhoA appears necessary for platelet-induced PMN chemotaxis *in vitro* downstream of specific $P2Y_1$ receptor activation with the $P2Y_1$ agonist MRS2365.

The impact of RhoA phosphorylation at the Serine 188 residue remains unclear. On the one hand, phosphorylation of S188 is believed to inactivate RhoA (Ellerbroek et al., 2003; Rolli-Derkinderen et al., 2010), trapping it in a complex with guanine dissociation inhibitor α (GDIα), locking the inactive RhoA within the cytosol. However, phosphorylation of RhoA has not been shown to induce full aggregation in response to ADP or other primary agonists.

Interestingly, recent studies have demonstrated that this phosphorylation mediated inactivation of RhoA induces the dissociation of Rac1 from GDIα allowing translocation of Rac1 from the cytosol to the membrane (**figure 6.2**). This allows its activation by Rac guanine exchange factors (Rac-GEFs) including Vav proteins or P-Rex proteins, thus initiating the Rac1 signalling pathway (Rolli-Derkinderen et al., 2010; Aslan et al., 2011). Indeed, full platelet Rac1 activation requires direct Gq signalling through receptors as stimulation with ADP or combined ADP and TxA_2 treatments fail to activate Rac1 in the absence of Gαq receptors (Rolli-Derkinderen et al., 2010). Furthermore Rac activation has previously been shown to be activated via the $P2Y_1$ receptor independent of the $P2Y_{12}$ and $P2X_1$ receptors (Soulet et al., 2005; Aslan and McCarty, 2013).

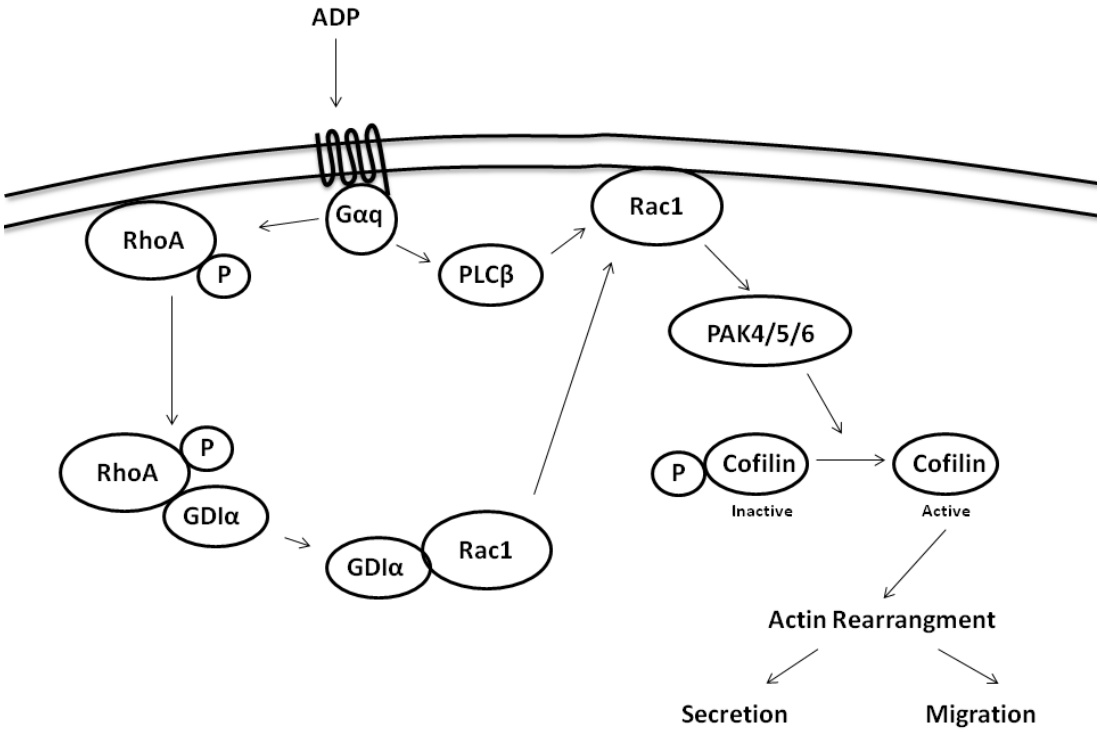


Figure 6. 2. RhoA mediated activation of Rac1

The Rac signalling pathway has also been implicated in the modulation of cell adhesion and migration in vascular smooth muscle cells (VSM) and human cancer cells (Rolli-Derkinderen et al., 2010; Valderrama et al., 2012; Carr et al., 2013). Rac1 signalling has also been shown to play a key role in the regulation of pulmonary neutrophil infiltration and tissue injury through regulating CXC chemokines (i.e. CXCL1 and CXCL2) and TNFα production in a murine model of abdominal sepsis (Hwaiz et al., 2013). Indeed, Rac1 inhibition was shown to decrease neutrophil accumulation in the lung, histology scores and Mac-1 expression on neutrophils, expression which is typically upregulated by CXC chemokines (Hwaiz et al., 2013). The apparent $P2Y_1$ dependent activation of Rac1, and the involvement of Rac1 in cell adhesion and migration demonstrates a potential separate pathway by which $P2Y_1$ receptor stimulation in inflammation may initiate

the inflammatory response without triggering full platelet aggregation. However these studies have demonstrated that inhibition of a RhoA dependent kinase as well as direct inhibition of RhoA inhibits pulmonary leukocyte recruitment *in vivo* as well as platelet-dependent leukocyte migration *in vitro*. It is therefore important to consider alternative mechanisms, as RhoA activation has also been implicated in inflammatory processes (Li et al., 2013; Ridley, 2013b; Sauzeau et al., 2013). Indeed, Platelets isolated from RhoA deficient mice demonstrate a normal phenotype under normal conditions. However, shape change was completely abolished to G_{13} agonists and severely impaired to G_q agonists such as ADP. Granule secretion to a wide range of platelet agonists is also severely impaired in RhoA deficient mice, as demonstrated by significant decreases in P-selectin expression following ADP stimulation and significantly increased bleeding times (Pleines et al., 2012). Rho and Rho associated kinase activation has previously been detected downstream of $P2Y_1$ signalling in vascular myocytes (Sauzeau et al., 2013).

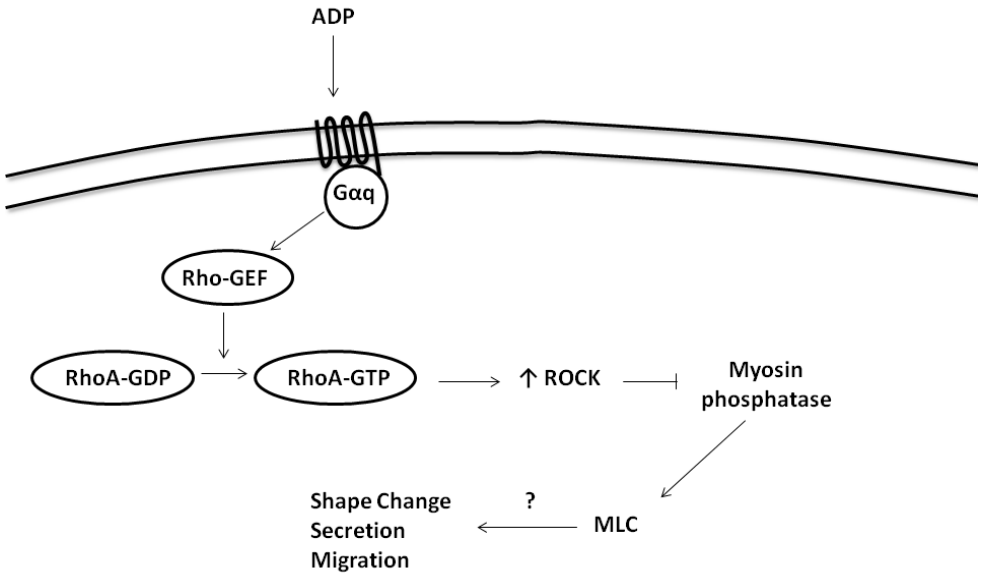


Figure 6. 3. $P2Y_1$ mediated activation of RhoA

It is also possible that the elevation in RhoA phosphorylation at S188 that has been detected 6 hours post allergen challenge is an indirect result of increased RhoA activity as described by Rho dependent activation of ROCK. If Rho activity is elevated post allergen challenge, over time a consequence of this will be elevations in pRhoA for its subsequent inactivation, as has been observed in this model of allergic inflammation. RhoA activation downstream of Gαq has previously been shown to be independent of PLCβ activation and is suspected to be activated though activation of Rho-guanine nucleotide exchange factors (Rho-GEFS) (Pleines et al., 2012), whilst RhoA mediated platelet shape change is known to function through inhibitory actions of MLC phosphatase allowing promoting rearrangement of the actin cytoskeleton (Pleines et al., 20120) (**figure 6.3**). However, whilst it is possible that rearrangement of the actin cytoskeleton through regulation of MLC prepares centralised granules for secretion the signalling mechanisms behind granule secretion remains unclear.

Within this context, the role of a ROCK inhibitor GSK429286 and the RhoA inhibitor C3 exoenzyme have been used to inhibit Rho activity both in the *in vitro* model of platelet-induced-PMN migration, as well as in the murine model of allergic pulmonary inflammation. In both cases, GSK429286 demonstrated significant inhibition of leukocyte recruitment both *in vitro* and *in vivo,* whilst C3 exoeznzyme and GSK429286 inhibited $P2Y_1$ specific stimulation of platelet induced PMN cell migration *in vitro*. Importantly, reinfusion of GSK429286 treated platelets into busulfan induced thrombocytopenic mice confirmed that platelet specific RhoA activity was involved. It is important however to clarify the exact mechanism by which modulation of RhoA signalling is involved in inflammatory cell recruitment, as both Rac1 and RhoA activation both exhibit the potential to increase cell motility

and migration. It is therefore important to investigate the activity of both platelet RhoA and Rac1 in allergen sensitised and challenged mice and the effects of the $P2Y_1$ specific receptor antagonist MRS2500 has on their activity states.

Nevertheless, this research suggests that the mechanism of platelet activation as a result of inflammatory stimuli, whilst requiring addition ADP stimulation of platelet $P2Y_1$ receptors is fundamentally different to mechanisms of platelet activation to 'classical platelet agonists' in hemostasis where additional co-stimulation of the $P2Y_{12}$ receptors by ADP is critical (Gachet, 2006, 2008). Modulation of RhoA signalling downstream of platelet $P2Y_1$ receptors therefore demonstrates a potential signalling pathway differentiating platelet activation towards inflammatory stimuli over classical platelet agonists.

6.2 Describing a Potential Dichotomy in Platelet Function

In Chapter V, experiments were performed to investigate the potential role of differential stimuli on platelet functions. Platelets express a large array of different receptors including receptors for hemostatic stimuli PAR1, PAR4, α2A adrenoceptor, β2 adrenoceptor, TPα, $P2Y_1$, $P2Y_{12}$ and $P2X_1$ (Quinton et al., 2004; Gachet, 2006, 2008; Li et al., 2010; Soh et al., 2010). Platelets also express a large array of receptors for inflammatory stimuli including the f-MLP receptor FPR (Czapiga et al., 2005), the LPS receptor TLR-2, TLR-4 and TLR-9 (Aslam et al., 2006; Semple and Freedman, 2010), the IL-1β receptor IL-1R1 (Brown et al., 2013), 5-HT2A (Li et al., 2010), the SDF-1α receptor CXCR4, the MDC receptor CCR4, the MIP-3 receptor CCR1, and the eotaxin receptor CCR3 (Clemetson et al., 2000) (**figure 6.4**). This produces scope for large differences in platelet activation leading to potential differences in platelet function. Chapter V describes the differential

impact of both inflammatory and haemostatic parameters of platelet function using inflammatory stimuli (SDF-1α, MDC, f-MLP, IL-1β, 5-HT, LPS) and hemostatic stimuli (Adrenaline, ADP, U46619 and TRAP1).

Using an *in vitro* assay of platelet chemotaxis, it was identified that stimulation of platelets with the chemokines SDF-1α, MDC and f-MLP induced significant chemotaxis compared to negative controls, a process that was not replicated by any hemostatic stimuli (Adrenaline, ADP, U46619, TRAP 1) or other inflammatory stimuli (IL-1β, 5-HT, LPS). In contrast however, IL-1β, 5-HT and LPS all demonstrated a potential (albeit not significant) increase in platelet-leukocyte conjugation, importantly none of the tested inflammatory stimuli were able to induce platelet aggregation.

All haemostatic stimuli induced significant platelet aggregation, significant elevations in platelet P-selectin expression and significant increases in the formation of platelet leukocyte conjugates, however none were capable of inducing platelet migration. This suggests fundamental differences in platelet function depending on which platelet receptors are stimulated. As expected, the haemostatic stimuli studied (ADP, Adrenaline, U46619 and TRAP-1) all induced significant platelet activation through stimulation of Gαq coupled receptors (ADP, U46619 and TRAP-1) (Gachet, 2006; Li et al., 2010; Soh et al., 2010) or the Gαs (adrenaline) (Rivera et al., 2009) inducing granule secretion and positive feedback of platelet activation through further release of platelet agonists. This was confirmed by the observed increases in P-selectin expression and formation of platelet-leukocyte conjugates following stimulation with ADP, TRAP, U46619 and adrenaline in agreement with the literature (Storey et al., 2002; Leon et al., 2004; Evangelista et al., 2005; Franks et al., 2010; Totani and Evangelista, 2010).

Whilst IL-1β, 5-HT and LPS demonstrated a potential increase in the formation of platelet leukocyte conjugates, this rise was not significant. This suggests that the current assay of platelet-leukocyte aggregate formation is not sensitive enough to detect the more subtle changes potentially induced by the weaker inflammatory stimulation of platelets. That said, the inability of IL-1β and LPS to induce an increase in P-selectin expression is not surprising. Previous studies investigating LPS and IL-1β stimulation of platelets have confirmed platelet activation post stimulation, but have demonstrated that this activation is not characterised by elevations in P-selectin expression (Schaufelberger et al., 1994; Brown et al., 2013) implying that platelet α-granules are not being secreted from the platelet. Indeed, previous work in murine platelets has demonstrated the LPS stimulation of platelets operates through an IL-1β autocrine loop resulting in the production of IL-1β mRNA amplifying platelet activation through ligation of TLR4 (Brown et al., 2013). Additional studies have also demonstrated potentiation of aggregation towards collagen and fibrinogen when co-stimulated with IL-1β (Beaulieu et al., 2014), further adding to the possibility of receptor cross-talk in the inflammatory actions of platelets.

The ability of the chemokines SDF-1α, MDC and f-MLP to induce platelet migration is consistent with reports in the literature suggesting platelets can undergo chemotaxis (Czapiga et al., 2005; Kraemer et al., 2010). Platelets express functional CXCR4, CCR4 and FPR receptors on their surface (Clemetson et al., 2012), whilst platelet activation has been demonstrated towards both SDF-1α and MDC (Clemetson et al., 2000; Abi-Younes et al., 2001; Gear, 2001). Importantly, these studies also demonstrate increases in P-selectin expression (Gear, 2001), with f-MLP stimulation inducing Ca^{2+} mobilisation in the platelet (Czapiga et al., 2005). Here, no

elevation in P-selectin was detected following stimulation with SDF-1α, MDC or f-MLP. It is possible this difference is a result in different protocols for P-selectin measurement, as Gear *et al.* 2001 measured P-selectin expression following agitation, an additional variable capable of contributing to platelet activation (Gear, 2001). It is also possible that the measurement of platelet activation through quantification of P-selectin expression is not a suitable assay as small elevations may not be detectable. Therefore, other assays investigating markers of platelet activation should be considered including measurements of platelet shape change, Ca^{2+} mobilisation and potentially CD40L expression. The suggestion that the assay of P-selectin expression demonstrates poor sensitivity as a marker of chemokine induced platelet activation is further supported by the ability of these chemokines (SDF-1α, MDC and f-MLP) to induce platelet migration. Indeed previous studies have demonstrated platelet migration to f-MLP and SDF-1α (Czapiga et al., 2005; Kraemer et al., 2010), thus demonstrating functional activity of platelets following stimulation by chemokines. Importantly, platelets stimulated with SDF-1α, MDC and f-MLP, whilst inducing significant chemotaxis had no effect on platelet aggregation, highlighting a potential mechanism by which platelet activation by inflammatory stimuli differs from platelet activation through hemostatic stimuli. This is interesting as chapter IV depicts a mechanism through ADP stimulation of the $P2Y_1$ receptor triggering inflammatory events (pulmonary leukocyte recruitment) without proceeding through to platelet aggregation, in agreement with heightened levels of platelet activation markers in asthmatic patients with a phenotype of mild hemostatic defects (Moritani et al., 1998; Sullivan et al., 2000; Pitchford et al., 2003a; Kowal et al., 2006). Given that ADP receptor $P2Y_1$ and the chemokines SDF-1α and MDC all signal through receptors coupled to the $G_{\alpha q}$ G protein (Gear, 2001;

Gachet, 2006; Clemetson et al., 2012), it is possible that co-operation between the signalling pathways exists. Indeed, studies have previously demonstrated that chemokine dependent aggregation or adhesion of platelets under flow conditions was dependent on low levels of primary agonists such as ADP, at sub-threshold concentrations required for aggregation alone (Gear, 2001). This being considered, initial preliminary experiments were performed investigating potential synergy between chemokine and ADP stimulation *in vitro.* Co-stimulation with ADP and MDC demonstrated synergy in the formation of platelet-leukocyte complexes in human whole blood potentially demonstrating a pathway by which inflammatory receptors combined with the $P2Y_1$ receptor could contribute to inflammatory actions of platelets without platelet aggregation. In chapter IV, inhibition of ROCK was demonstrated to inhibit platelet-induced PMN chemotaxis *in vitro* whilst also inhibiting pulmonary leukocyte recruitment *in vivo*. Given the ability of the $G_{\alpha q}$ coupled $P2Y_1$ receptor to modulate the signalling of RhoA seen here and reported RhoA signalling downstream of $G_{\alpha q}$ coupled receptors (Mong and Wang, 2009; Pleines et al., 2012; Aslan and McCarty, 2013), it is possible that chemokine receptors such as the SDF-1α receptor CXCR4 and the MDC receptor CCR4 also signal through RhoA and synergise with subthreshold concentrations of ADP stimulating the high affinity $P2Y_1$ receptor inducing platelet activation which is fundamentally different to aggregation with no secondary phase aggregation observed (**figure 6.1**). Therefore, the impact of chemokine stimulation of RhoA activity, or investigations should be performed to detect if inflammatory mediator stimulation of platelet migration is sensitive to inhibition of ROCK.

6.3 Future Work

In chapter IV, a particular role of the ADP activated $P2Y_{1,}$ but not the $P2Y_{12}$ or $P2X_{1,}$ receptors was discovered to be critical in pulmonary leukocyte recruitment *in vivo*, platelet-leukocyte conjugation *in vitro* and platelet-induced-PMN migration *in vitro*. Whilst modulation of RhoA signalling downstream of the $P2Y_1$ receptor is evident, it still remains unclear as to why activation of the $P2Y_1$ receptor under inflammatory conditions does not signal the same way as activation to haemostatic stimuli demonstrated by granule secretion and subsequent activation of the $P2Y_{12}$ receptor critical in the amplification of $P2Y_1$ initiated platelet aggregation. Therefore, further work needs to investigate in more detail how RhoA activity is modulated following allergen challenge and the effects on other signalling events mediated by the $P2Y_1$ receptor. Furthermore, if RhoA is becoming activated which specific proteins are involved? Is the G_q associated G protein directly responsible for the production of active GTP bound RhoA? If so, is this process independent of $G_{\alpha q}$ activation of PLCβ and subsequent elevations in intracellular Ca^{2+}, PKC activation and α-granule secretion?

In the *in vitro* model of platelet-induced PMN chemotaxis, platelets become activated following stimulation with 0.1μM ADP and chemokine (MDC, SDF-1α, f-MLP). 0.1μM ADP is incapable of inducing platelet aggregation and is such considered to be a sub-threshold concentration. However, in combination with additional chemokine stimulation, significant platelet-induced leukocyte chemotaxis is observed. Furthermore, costimulation of platelets with MDC and 10μm ADP demonstrates elevated platelet-leukocyte conjugation greater than the summation of

stimulation with either MDC or ADP alone. When this is combined with evidence in the literature demonstrating significant potentiation of platelet function induced by MDC, TARC and SDF-1α when constimulated with low dose ADP (0.1μM) (Gear, 2001), it becomes clear that there is potential co-operation between ADP receptors and other receptors such as the chemokine receptors. However, work needs to be investigated on a range of different receptors to investigate if co-operation is limited to G_q coupled receptors such as the CXCR4 and CCR4 receptors or whether it is a more generic phenomenon involving other inflammatory receptors such as TLR's, 5-HT receptors, IL-1R1 etc. Therefore using a similar experimental plan to those used in experiments described in chapter V, co-operation of low dose ADP with the inflammatory stimuli SDF-1α, MDC, IL-1β, f-MLP, 5-HT, LPS needs to be investigated.

Finally, at the outset of this research the aim was to investigate the differential role of platelet ADP receptors in pulmonary leukocyte recruitment. This mechanism of platelet involvement in inflammation may be more widespread than solely allergen induced leukocyte recruitment. Indeed, platelets have previously been implicated in non-allergic conditions of inflammation including COPD, RA, IBS and atherosclerosis (Ott I, Neumann FJ, Gawaz M, Schmitt M, 1996; Ferroni P, Basili S, Martini F, Vieri M, Labbadia G, Cordova C, Alessandri C, 2000; Bunescu et al., 2004). Platelets have also more recently been implicated in inflammation associated with bacterial infection. Indeed, platelet dysfunction has been observed in cystic fibrosis (CF), with increased platelet activation observed towards gram-negative infection with increased elevations in platelet derived mediators, platelet-monocyte interactions, platelet-neutrophil interactions and P-selectin expression (O'Sullivan et al., 2005; O'Sullivan and Michelson, 2006). It is therefore important to investigate

whether the $P2Y_1$ specific role in pulmonary leukocyte recruitment observed in this murine model of acute allergic inflammation is replicated in additional models such as LPS induced pulmonary leukocyte recruitment and in a model of respiratory infection with *Pseudomonas aeruginosa* in the rat mimicking pulmonary inflammation typical of CF (Growcott et al., 2011).

6.4 Final Remarks

The conjugation of platelets with leukocytes and their subsequent recruitment to the lungs is dependent on the expression of platelet P-selectin and its interactions with its counter ligand PSGL-1 (Sanctis et al., 1997; Lukacs et al., 2002; Pitchford et al., 2005), furthermore a number of studies have demonstrated that activation of both the $P2Y_1$ and $P2Y_{12}$ receptors induced elevations in P-selectin expression through granule secretion (Storey et al., 2002; Leon et al., 2003). It was therefore somewhat surprising that in these studies $P2Y_{12}$ receptor activation does not appear to be involved in the P-selectin-dependent mechanism of pulmonary leukocyte recruitment. It stands to reason that the mechanism of platelet activation by inflammatory mediators is fundamentally different to the mechanism of platelet activation induced by the 'classical' platelet agonists, where co-activation of the $P2Y_{12}$ receptor by ADP is involved, even when the end result (P-selectin) is similar but ultimately results in differences in platelet function. This ability of inflammatory stimuli to induce platelet activation without altering haemostasis highlights a fundamental dichotomy in platelet activation (Page, 1988). This has far reaching implications for a wide range of inflammatory disorders associated with heightened levels of platelet activation including asthma, allergic rhinitis and eczema.

Chapter VII

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